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Name of Candidate: Bethanie L. Morrison
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June 19, 2009

Dissertation and Abstract Approved:

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Dr. Mary Lou Cutler Department of Pathology Dissertation Advisor	✓	___
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14. ABSTRACT

The degree of differentiation of mammary epithelial cells is dependent on their response to basement membrane and stromal protein-induced signals. Connective Tissue Growth Factor (CTGF/CCN2), a known stromal mediator, is highly upregulated by dexamethasone and is required for the lactogenic differentiation of mouse mammary epithelial cells. Using a Tet-off system to overexpress CTGF/CCN2 in the HC11 cell background, we found that its elevated expression enhanced multiple markers of lactogenic differentiation, including β-casein transcription, mammosphere formation, and stabilization of Stat5 activity. Elevated levels of CTGF/CCN2 partially abrogated the requirement of matrix proteins for the transcription of β-casein, suggesting that CTGF/CCN2 contributes to lactogenic differentiation through a regulation of cell:matrix adhesion.

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Bethanie L. Morrison
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Abstract

Title of Dissertation:	Connective Tissue Growth Factor (CTGF) as a regulator of lactogenic differentiation.
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Dissertation directed by:	Mary Lou Cutler, Ph.D Professor Department of Pathology Uniformed Services University of the Health Sciences

The degree of differentiation of mammary epithelial cells is dependent on their response to basement membrane and stromal protein-induced signals. Connective Tissue Growth Factor (CTGF/CCN2), a known stromal mediator, is highly upregulated by dexamethasone and is required for the lactogenic differentiation of mouse mammary epithelial cells. Using a Tet-off system to overexpress CTGF/CCN2 in the HC11 cell background, we found that its elevated expression enhanced multiple markers of lactogenic differentiation, including β-casein transcription, mammosphere formation, and stabilization of Stat5 activity. Elevated levels of CTGF/CCN2 partially abrogated the requirement of matrix proteins for the transcription of β-casein, suggesting that CTGF/CCN2 contributes to lactogenic differentiation through a regulation of cell:matrix adhesion. CTGF/CCN2 contributed to the survival of HC11 cells under serum-free conditions during which epithelial cells typically undergo anoikis. HC11 cells expressing CTGF/CCN2 exhibited an increase in the level of α6 and β1 integrins and downstream survival signaling mediators including phospho-FAK, phospho-Akt, and cyclin D1.

Elevated CTGF/CCN2 levels increased the formation of focal adhesion complexes, as viewed by immunofluorescence, and increased expression levels of focal adhesion-related adaptor, structural, and kinase proteins including parvin, p130cas, paxillin, Src, vinculin, and integrin-linked kinase. HC11 cells also display enhanced adhesion to CTGF/CCN2, and this increase is inhibited in the presence of function-blocking antibodies to α6 and β1 integrins. These results demonstrate that the mechanism by which CTGF/CCN2 contributes to lactogenic differentiation is via activation of α6β1-mediated adhesion complexes and integrin-dependent signaling pathways.

CONNECTIVE TISSUE GROWTH FACTOR (CTGF) AS A REGULATOR OF LACTOGENIC DIFFERENTIATION

By

Bethanie L. Morrison

Dissertation submitted to the Faculty of the Graduate Degree Program in Molecular Pathobiology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
2009

Acknowledgements and Dedication

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Abbreviations

AKT	v-akt murine thyoma viral oncogenes homolog	ATP	Adenosine triphosphate
ATF2	Activating transcription factor 2	BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2	BM	Basement membrane
BSA	Bovine serum albumin	bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate	C/EBPβ	Ccaat-enhancer binding protein β
CCD	Charge coupled device	Cdk	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation	CMV	Cytomegalovirus
CoRE	Composite response element	CTGF	Connective Tissue Growth Factor
CYR61	Cysteine-rich angiogenic inducer 61	DAB	Diaminobenzidine
DDR1	Discoidin domain receptor 1	DIP	Dexamethasone Insulin Prolactin
DNA	Deoxyribonucleic acid	DOC	Deoxycholate
DOX	Doxycycline	DTT	Dithiothreitol
ECM	Extracellular matrix	EGF	Epidermal growth factor
ER	Estrogen receptor	ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase	FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate	GFP	Green fluorescence protein
GR	Glucocorticoid receptor	GRE	Glucocorticoid response element
HA	Hemagglutinin	HEPES	4-(2-hydroxyethyl)-1-peperazineethanesulfonic acid
IGF	Insulin-like growth factor	ILK	Integrin-linked kinase
JAK2	Janus kinase 2	JNK	c-jun terminal kinase
LAP	Liver-enriched activator protein	LIP	Liver-enriched inhibitory protein

LDL	Low-density lipoprotein	LM-1	Laminin-1
LRP	Low-density lipoprotein receptor-related protein	LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase	MEC	Mammary epithelial cell
MGF	Mammary gland factor	MMP	Matrix metalloproteinase
MOI	Multiplicity of infection	MTT	3—(4,5-Dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B	NOV3	Nephroblastoma overexpressed 3
P90RSK	p90 ribosomal S6 kinase	PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffered saline	PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor	PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinase	PKC	Protein kinase C
PLK3	Polo-like kinase 3	PR	Progesterone receptor
PTP1B	Protein tyrosine phosphatase 1B	PTPRκ	Protein tyrosine phosphatase receptor type kappa
PVDF	Polyvinylidene fluoride	RAC	Ras-related C3 botulinum toxin substrate
RAD	Glycine-Arginine-Alanine-Asapartate-Serine-Proline	RELB	v-rel reticuloendotheliosis viral oncogenes homolog B
RGD	Glycine-Arginie-Glycine-Aspartate-Asparagine-Proline	RHO	Ras homolog
RNA	Ribonucleic acid	RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation	SRC	v-Schmidt-Ruppin A-2 sarcoma viral oncogene homolog
SSC	Saline sodium citrate	STAT5	Signal transducer and activator of transcription 5
TEB	Terminal end bud	TGFβ	Transforming growth factor β

TIMP3 Tissue inhibitor of metalloproteinase 3

TNFRSF12a Tumor necrosis factor receptor superfamily 12a

TUNEL Terminal dUTP nick-end labeling

vWC von Willebrand type C

WISP Wnt-induced secreted protein

YY-1 Yin-yang 1

TNF Tumor necrosis factor

TSP Thrombospondin

VEGF Vascular endothelial growth factor

WAP Whey acidic protein

WNT Wingless-type MMTV integration site

Chapter 1: Introduction

Overview

The work in this thesis addresses the mechanism of Connective Tissue Growth Factor (CTGF/CCN2) in mouse mammary epithelial cells during lactogenic differentiation. Our lab has worked extensively on the mechanisms involved in lactogenic differentiation (31, 82, 270, 271). Recently, a mouse developmental microarray analysis was performed on HC11 mouse mammary epithelial cells that had been induced to differentiate, and it was found that CTGF/CCN2 was one of the most highly upregulated genes in the differentiating cells compared to the non-differentiating control (271). This was a significant finding, as it has recently been shown that CTGF/CCN2 is associated with metastatic breast tumors (37). This was an interesting finding as well because CTGF/CCN2 is primarily regarded as a regulator of bone development and fibrosis (68, 118). The function of CTGF/CCN2 in lactogenic differentiation was unknown. We have recently established that elevated expression of CTGF/CCN2 can enhance the transcription of milk proteins, and that it is required for lactogenic differentiation (271). One of the main requirements for the transcription of milk proteins is the interaction of mammary epithelial cells with the surrounding basement membrane (33). This interaction is known to be mediated through β1 integrin-containing complexes on the surface of the epithelial cells (2). CTGF/CCN2 is a matrix-associated protein (35) that interacts with integrin complexes in many cell types, including β1 integrin-containing complexes (154, 192). Thus, the hypothesis for this study is that CTGF/CCN2 contributes to lactogenic differentiation by acting as a

regulator of cell:matrix adhesion through the activation of β1 integrin-containing complexes.

In order to fully comprehend lactogenic differentiation and the various mechanisms involved, an extensive overview of mammary gland development is provided in this introduction, including the factors and regulations relevant to the current research. A broad introduction to CTGF/CCN2 is also provided, as well as its regulation and diverse functions in various tissues and cell types. It is important to understand the molecular mechanisms involved in the development of the mammary gland as well as the different mechanisms by which CTGF/CCN2 is regulated and functions so as to fully comprehend the rationale behind this study. This investigation into the role of CTGF/CCN2 in mouse mammary epithelial cells has resulted in the conclusion that CTGF/CCN2 acts as a regulator of integrin-mediated adhesion and survival, functions which allow for the transcription of milk proteins during lactogenic differentiation. The comprehension of the mechanism of CTGF/CCN2 is critical to understanding the process of lactogenesis, as well as to the understanding of the possible role for CTGF/CCN2 in metastatic breast cancer.

Mammary Gland Development

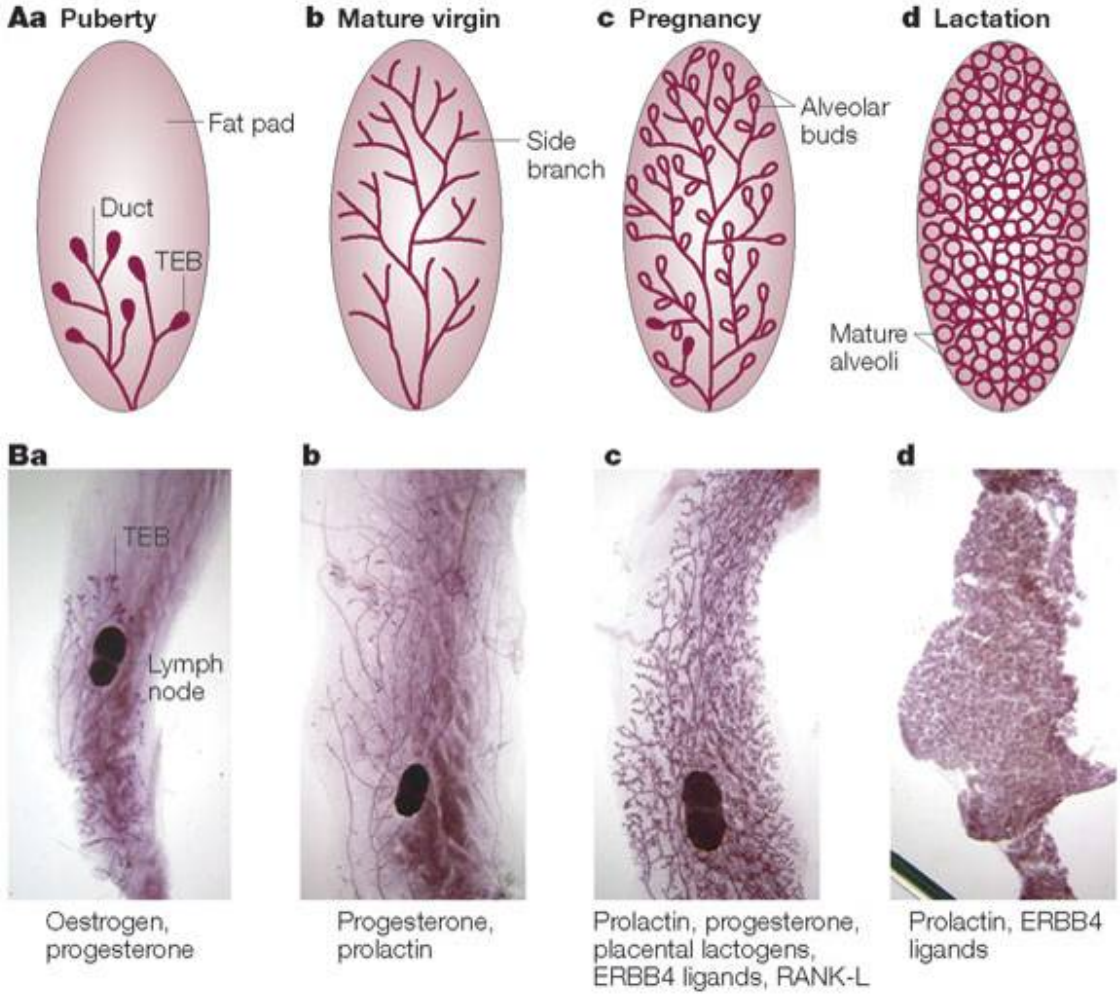
The mammary gland undergoes cycles of pubertal maturation, pregnancy, lactation, and a regression phase known as involution. While a rudimentary mammary gland structure is present at birth, the majority of glandular development takes place after birth. Further maturation of the gland is hormone-dependent and begins at puberty.

Morphological changes of the pre-pubescent mammary gland

The virgin mammary gland, which has not yet undergone pregnancy or lactation, consists of ducts extending from the nipple through the mammary fat pad to the functional terminal end points referred to as the terminal end buds (TEB) in mice, or alveoli in humans. Mouse and human mammary glands are very similar in structure and function. A primary difference between the mammary gland of the two species is the TEB unit. The TEB is a single bulbous structure that develops into a more complex collection alveolar structures known as the lobulo-alveolus. In the human, multiple bulbous ends are present on multiple ducts, and these grow and expand during pregnancy (277). The work described in the ensuing chapters uses the cells of the mouse mammary gland as a model. The morphological changes that occur during the development of the mouse mammary gland are depicted in **Figure 1**.

The TEBs consist of two main cellular layers: an inner layer of luminal secretory epithelial cells and an outer layer of contractile myoepithelial cells that surround a central hollow lumen. Surrounding the TEB is a basement membrane layer of highly cross-linked extracellular matrix (ECM) proteins, laminin being the predominant protein. The basement membrane proteins are secreted primarily by the outer layer of myoepithelial cells. Outside of the basement membrane lies the interstitial ECM, which is largely composed of collagen types I and III, as well as other common ECM proteins such as fibronectin, laminin, and tenascin. The rudimentary structures that are present at birth grow at the same rate as the animal until the onset of puberty (112).

Figure 1. Mouse mammary gland development. *Adapted from Hennighausen et al.(104)*. **A**. Schematic drawings of the development of the mouse mammary gland from puberty through lactation. **B**. Whole mounts of mouse mammary glands at the same stages of development as depicted in part A. (**a**) Rudimentary ductal structures and terminal end buds begin to form due to the cyclical production of estrogen and progesterone. (**b**) Ductal branching is enhanced and side branching occurs as the gland matures. (**c**) At the onset of pregnancy, alveolar buds become differentiated and functional as the gland prepares for lactation. (**d**) The alveolar sacs fill with milk at parturition. Milk release is initiated by suckling and is terminated at the conclusion of weaning.



Morphological changes and hormonal regulation of the mature virgin mammary gland

Pubertal development is morphologically initiated by the invasion of ductal epithelial cells into the surrounding stroma, which results in the recruitment of the ECM components and the formation of a basement membrane around the TEBs (277). Predominant cell types present in the mammary stroma include fibroblasts, immune cells, and adipocytes. Adipocytes are the most abundant stromal cells in the mammary gland, however, they are the least studied regarding their functionality during development (47). Fibroblasts are the most functional component of connective tissue and they are the primary producers of matrix proteins such as fibronectin and laminin. The immune cells present in the stroma typically include the primary migratory cells of the immune system such as macrophages, eosinophils, neutrophils, and mast cells. Macrophages are the most abundant type of immune cell in the stroma surrounding the TEBs of the developing gland due to their phagocytic properties that rid the gland of debris and apoptotic cells.

The invasion by the ductal epithelium of the mature basement membrane and periductal stroma is a process referred to as branching morphogenesis. The exact mechanism of branching morphogenesis remains unclear, though it is known to involve the breakdown of the ECM proteins by matrix metalloproteinases (MMP) (279). MMP-3, specifically, is thought to regulate side branching (278).

The vast majority of the epithelial cells of the pre-pubescent gland express receptors for the ovarian hormones estrogen and progesterone; the cyclical production of which is responsible for the stimulation of ductal outgrowth and branching during

puberty. Estrogen is primarily responsible for the growth of ductal structures (180), while progesterone plays a major role in the expansion of the TEBs or alveolar structures (294). Progesterone receptors (PR) are typically found on proliferating cells of the TEB, yet progesterone can act in a paracrine manner such that cells adjacent to PR-expressing cells are also stimulated to undergo proliferation, probably via the activation of a non-PR-mediated signal transduction pathway, such as NF-κB (25). Hormones released from the pituitary gland, such as growth hormone (GH), also play roles in ductal morphogenesis during puberty. In ovariectomized mice, treatment with estrogen restores the TEB formation that had been lost (52), while the treatment with growth hormone rescues the TEB formation in hypophysectomized mice (136). At the conclusion of puberty, the mature virgin mammary gland is full of primary and secondary ductal systems, the latter of which appear and disappear corresponding to the estrous cycle.

Morphological changes and hormonal regulation of the pregnant mammary gland

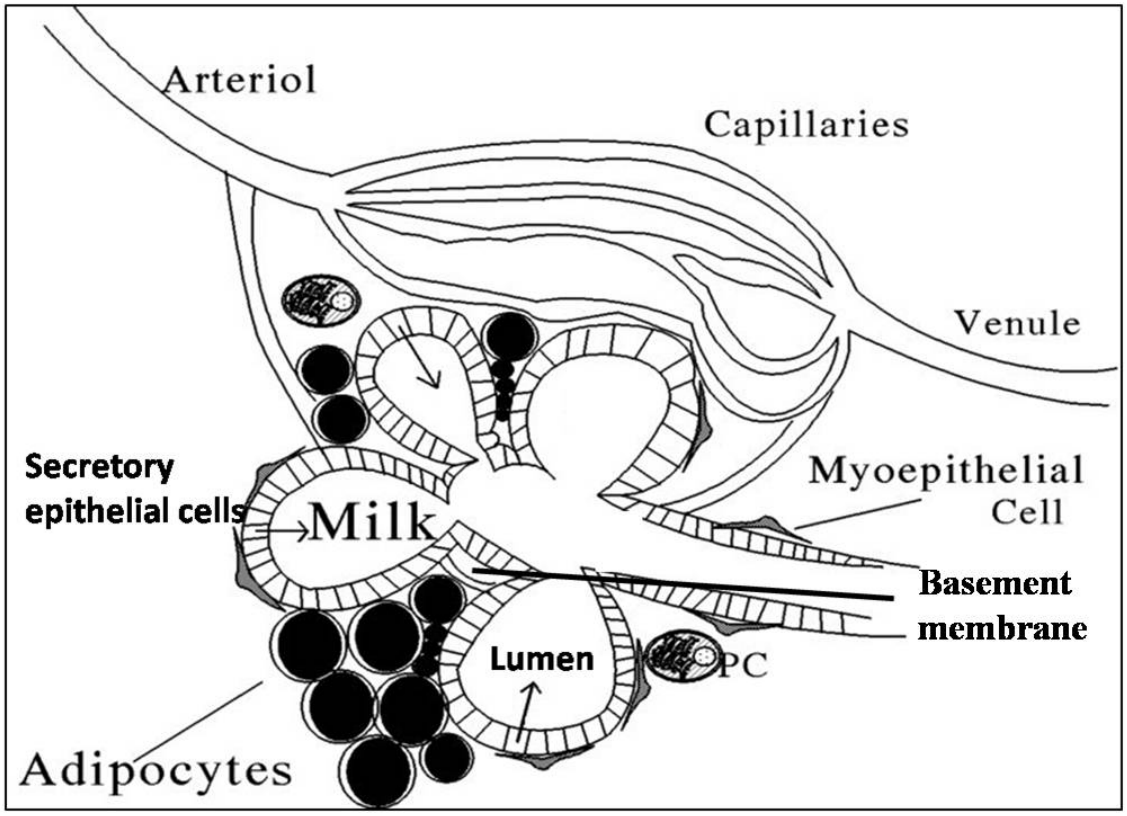
During pregnancy, lateral buds extend through the main ducts. These buds undergo massive proliferation and subsequent differentiation in order to fill the gland with lobuloalveolar structures that contain the secretory epithelial cells. Later in pregnancy, the alveolar complexes increase in number and complexity, and the cells lining the alveoli and small ducts mature, acquiring the ability to secrete milk. The milk secretion, however, is kept in check by high concentrations of circulating progesterone until the initiation of lactation (258).

At the onset of pregnancy, the anterior pituitary gland is stimulated to produce prolactin, a single peptide hormone that plays a significant role during both pregnancy

and lactation. Prolactin has two main functions in reproduction: the maintenance of the corpus luteum during early pregnancy and the development of the mammary gland throughout pregnancy. Through the preservation of the corpus luteum, prolactin ensures the secretion of estrogen and progesterone (83). Progesterone is also an important factor in regulating the morphological changes in the mammary gland during pregnancy. Progesterone receptor knockout studies in mice revealed that progesterone is required for the process of ductal side branching (113), while prolactin knockout studies revealed that it is required for alveologenesis during pregnancy (24). While the main sources of prolactin are the lactotroph cells of the pituitary, the production of prolactin by mammary epithelial cells has been reported, where it functions as a paracrine mediator of mammary epithelial cell development (17, 195).

The establishment of functional alveoli during pregnancy depends on the polarization of the luminal cells and the formation of junctions between them (12). This functional gland is depicted in **Figure 2**.

Figure 2. The developed mammary alveolar structure. *Adapted from http://mammary.nih.gov/reviews/lactation/Neville001/index.html.* Alveolar structures consist of hollow central lumens surrounded by a single layer of secretory epithelial cells. This layer of cells is attached to a basement membrane structure as well as a layer of myoepithelial cells. The myoepithelial cells contract as a result of the oxytocin released during suckling. This contraction causes physical stress on the epithelial cells, causing them to secrete their milk proteins.



Proteins required for the polarization of the cells and for the formation of tight junctions are regulated, in part, by adhesion-mediated signals. During pregnancy, the myoepithelial cell barrier of the TEB is stretched, thereby allowing many luminal cells to make direct contact with the basement membrane, directly altering cell adhesion and subsequent signaling. These changes orchestrate the massive tissue remodeling that occurs in preparation for lactation, resulting in a functional secretory gland (195).

Morphological changes and hormonal regulation of the lactating mammary gland

At parturition, a fall in progesterone levels, accompanied by the maintained elevation of prolactin, leads to secretory cell activation and lactation (189). After parturition, the act of suckling stimulates the release of oxytocin from the posterior pituitary gland, which causes the myoepithelial cell layer to constrict, forcing the milk out of the secretory cells and into the lumen of the alveolus and out through the ductal structures to the nipple. A diagram depicting hormonal control of alveolar architecture from puberty through parturition is shown in **Figure 3**. Multiple secretory processes are utilized in the epithelial cells of the lactating mammary gland: exocytosis, lipid synthesis and secretion, transmembrane secretion of ions and water, and transcytosis of extra-alveolar proteins such as immunoglobulins and albumins as well as hormones from the interstitial space.

The luminal epithelial cells are responsible for converting precursor proteins into milk components and transporting them into the lumen of the alveolar structures. The interaction between the epithelial cells and the stromal cells is an essential part of the secretory process. While the cues that come from this interaction may contain some cell-

specific components, most are of a general nature, mediating responses such as proliferation and survival. Aside from their contractile function, the physical and paracrine interactions between luminal and myoepithelial cells are critical for maintaining luminal cell polarity, as well as regulating proliferation and apoptosis (143). To ensure the properly polarized secretion of milk components, the formation of tight junctions between secretory cells is required. Main components of tight junctions, specifically occludin and ZO-1, are induced by both prolactin and glucocorticoids (240).

Successful lactation depends upon a pulsatile release of prolactin from the pituitary gland (282). While it is known that prolactin instructs the proliferation and the differentiation of the mammary epithelium through mechanisms specific to the prolactin receptor (26), evidence also suggests that prolactin-mediated signaling, similar to signaling resulting from adhesion to the basement membrane, activates transcriptional programs that are shared between several receptors, such as integrins (24). The mechanistic details of lactogenesis will be described in detail later in this introduction.

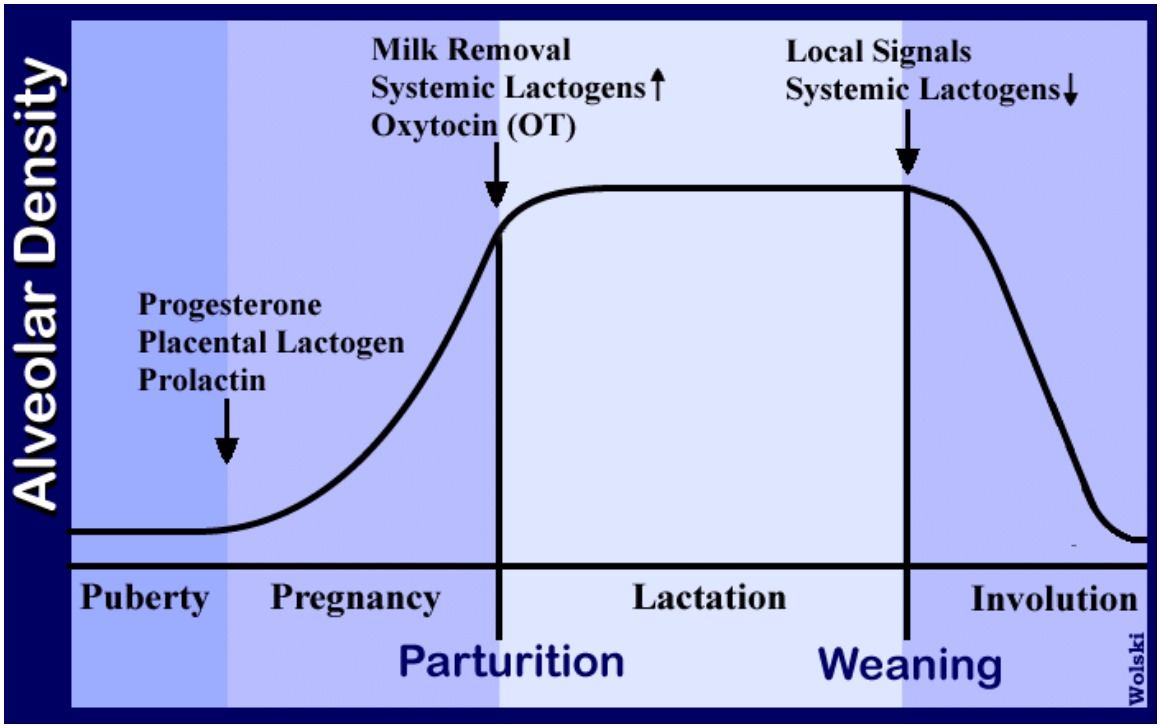
Morphological changes and hormonal regulation of the involuting mammary gland

At the conclusion of weaning, the lobuloalveolar compartment undergoes massive apoptotic cell death and tissue remodeling in order to restore the ductal architecture similar to that of the mature virgin gland. This process is referred to as involution. Involution can be broken into two main phases: the initial reversible phase, and the non-reversible phase. During the initial phase, which lasts approximately 48 hours, milk accumulation induces the upregulation of pro-apoptotic factors and the reduction of

survival factors. This phase can be reversed by re-suckling. The second phase is irreversible as cells undergo widespread apoptosis and tissue remodeling occurs.

A cluster study performed by Clarkson *et al*. (43) characterized the first 48 hours of the "involution switch," including the first 12 hours, 24 hours, 24 hour prolonged, and 48 hour responses. It was found that the initial 12 hour phase involves an increase of cell-death-promoting ligands and their receptors, pro-inflammatory cytokines, and neutrophil-attracting chemokines. The 24 hour gene cluster is characterized by an increase in the cell death pathway involving Vitamin D3, as well as an increase in cell-cell attachment proteins and integrins that are involved in the formation of hemidesmosomes between the myoepithelial cells and the basement membrane. The 24 hour prolonged response is a very acute phase displaying increases in genes that may be involved in the phagocytosis of apoptotic cells, a transforming growth factor β (TGFβ) response, and activation of the pro-apoptotic Bcl-2 family member, Bax. The 48 hour delayed response is characterized by apoptosis and tissue remodeling. At this point in the involution phase, the lumen of the alveolar structures is no longer detectable and the gland resembles a healing wound, including the massive infiltration of immune cells. Also during this phase, adipocytes repopulate the gland. After approximately one week of involution, the morphology of the mammary gland resembles that of the mature virgin.

Figure 3. Hormonal regulation of mammary alveolar architecture. *Adapted from http://mammary.nih.gov/reviews/development/Hennighausen001/index.html*. Mammary gland development during puberty is primarily due to the cyclical changes in ovarian estrogen and progesterone. The development of the alveolar structures begins at the onset of pregnancy, and is regulated by enhanced and sustained levels of progesterone, as well as placental lactogens and the pituitary hormone prolactin. At parturition, oxytocin causes milk removal, progesterone expression is decreased, and systemic lactogens now play a major role in the stabilization of the secretory structures. At the conclusion of weaning, systemic lactogens decrease and local signals are generated causing the alveolar structures to undergo apoptosis such that the gland reverts back to its pre-pregnancy structure.



Lactogenesis/Milk Protein Production

Hormonal regulation of milk protein gene expression

During pregnancy, the luminal secretory epithelial cells are induced to produce milk and whey proteins. The predominant milk proteins in all species are the caseins, and the primary whey protein is whey acidic protein (WAP). The genes encoding these proteins display both developmental and tissue-specific patterns of expression (108). Both caseins and whey proteins are used as molecular markers of functional differentiation in the mammary gland (256). The caseins are the most widely used proteins as markers of lactogenic differentiation in the mouse mammary gland.

The casein genes are encoded by a 250kb cluster on chromosome 5 of the mouse genome (215). The casein genes include αs1, β, γ, δ, and κ, with β-casein being expressed at the highest levels in the mouse (227). β-casein is arranged in a 3' → 5' transcriptional orientation. The regulation of the β-casein gene has been defined primarily *in vitro* by analyzing constructs in stably transfected mammary epithelial cells, including HC11 mouse mammary epithelial cells (228). This cell line was derived from the COMMA -1D line that originated from the mammary gland of a pregnant Balb/c mouse (115). These cells are used primarily to study the mechanism of lactogenic differentiation due to their ability to respond to lactogenic hormones and induce the transcription of the β-casein gene. Conveniently, HC11 cells also deposit their own laminin-rich matrix after being cultured for several days at confluence (33). The precise contribution of specific transcription factors and their binding sites can only be determined *in vivo* using transgenic and knockout mice, thus primary cultures from

knockout mice have been used to confirm the importance of these factors in the regulation of milk protein gene expression (163, 218, 232).

Studies in both HC11 cells and primary cells from various transgenic and knockout models have established that hormonal and developmental regulation of the β-casein gene requires a complex DNA element referred to as a composite response element (CoRE) (15). These CoRE units are defined as a cluster of transcription factor binding sites containing both positive and negative regulatory elements which integrate signal transduction pathways through protein:DNA and protein:protein interactions (121). The primary factors associated with activation of the β-casein CoRE include signal transducer and activator of transcription 5 (Stat5), glucocorticoid receptor (GR), and Ccaat enhancer binding protein β (C/EBPβ), while Yin Yang-1 (YY-1) associates with the CoRE as a negative regulator of gene expression (65, 151, 170, 211, 212, 228, 232, 267). Diagrams depicting the proximal promoter of β-casein and the involvement of the β-casein CoRE are shown in **Figure 3**. Interestingly, none of the transcription factors associated with the β-casein CoRE is mammary gland-specific or even restricted to the lactation phase of development.

Stat5 in lactogenesis

Originally identified in the sheep mammary gland as mammary gland factor (MGF) (268), Stat5 is the primary transcription factor associated with the activation of the β-casein gene. The activation of Stat5 occurs as the result of the activation of the prolactin receptor. During pregnancy, prolactin binds to the extracellular portion of the prolactin receptor and initiates events involving the activation of the cytoplasmic protein tyrosine

kinase of the Janus kinase family, Jak2 (4) and subsequent activation and nuclear translocation of Stat5. This pathway is depicted in **Figure 4**. Heterozygous knockout mice of both prolactin and the prolactin receptor display decreased milk protein gene expression and decreased mammary gland development (110).

Figure 4. The β-casein CoRE. (**a**) *Adapted from Biochemical Society Transactions www.biochemsoctrans.org Biochem. Soc. Trans. (2007) 35, 18-22.* Transcription of the β-casein gene requires the cooperative action of the chromatin remodeling machinery and transcription factors. (**b**) *Adapted from Kabotyanski et al. (125).* β-casein proximal promoter and binding sites for respective transcription factors.

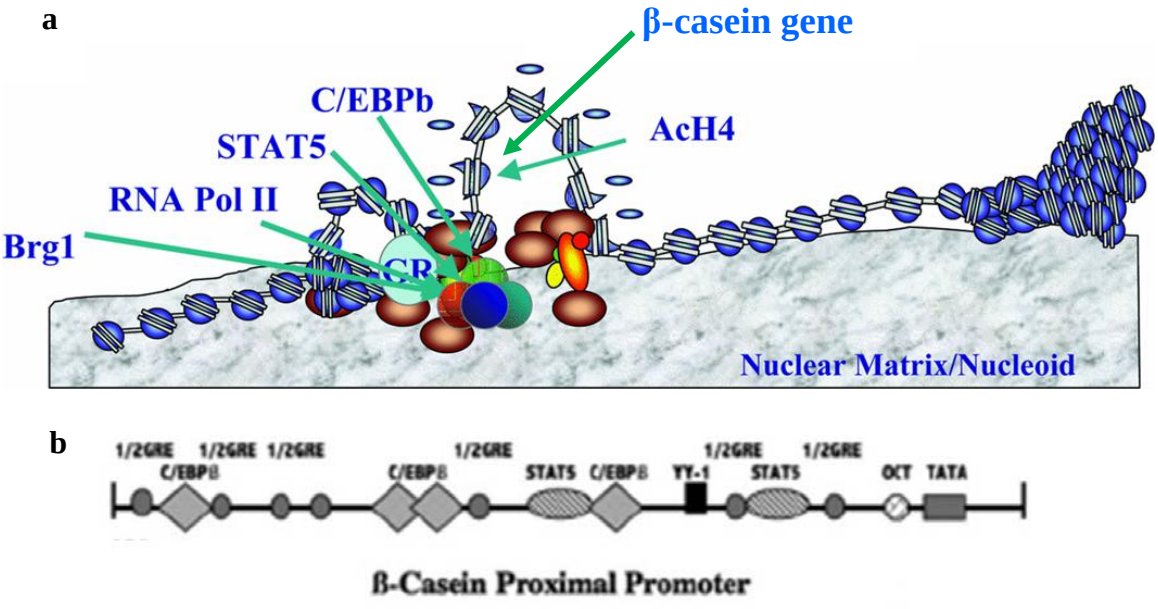
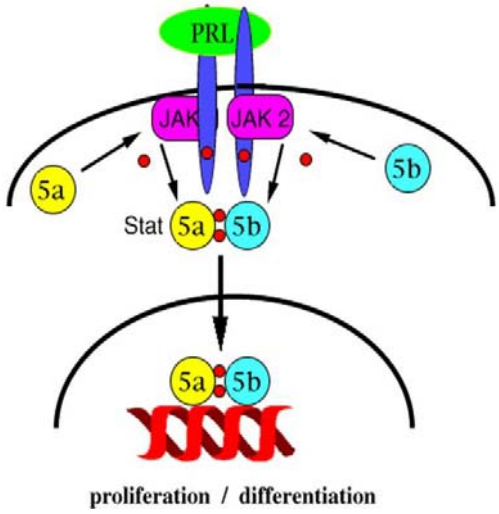


Figure 5. The prolactin receptor/Jak2/Stat5 signaling pathway. *Adapted from* http*://mammary.nih.gov/reviews/development/Hennighausen001/index.html.* Signaling through the prolactin receptor/Jak2/Stat5 pathway is initiated upon the binding of prolactin to the receptor, which is immediately followed by the phosphorylation and activation of the receptor. The activated receptor recruits Jak2, which becomes activated and recruits Stat5a and b. These proteins become phosphorylated, activated, and subsequently dimerized and translocated to the nucleus where the dimer is available for DNA binding activity.



In the absence of ligand, dormant Jak2 is associated with the prolactin receptor and is activated by transphosphorylation when two Jak2 proteins are brought together by ligand-induced dimerization of the prolactin receptor (220). Phosphorylated Jak2 is then able to tyrosine phosphorylate the prolactin receptor, which creates docking sites for proteins containing src homology 2 (SH2) domains, including Stat5 (205). The Stat5 proteins are tyrosine phosphorylated by Jak2, which leads to their dimerization and translocation to the nucleus. At the promoter, the Stat5 dimer binds to the gamma interferon activation sequence (GAS) of the β-casein promoter (92, 267). Stat5 may then be recycled back to the cytoplasm following inactivation by a nuclear tyrosine phosphatase such as the protein tyrosine phosphatase PTP1B (5, 179).

In the mouse, two Stat5 genes exist that are extremely similar and are probably the result of gene duplication, Stat5a and Stat5b. These proteins share 93% homology with the only major difference occurring in the transactivation domain (161). Mice lacking Stat5 gene expression (163) display decreased lobuloalveolar development and have the inability to lactate. Interestingly, Stat5b knockout mice do not have as severe developmental defects as do Stat5a knockout mice (253). Surprisingly, it has also been demonstrated that the absence of either Stat5a or Stat5b does not dramatically alter β-casein gene expression, suggesting that both forms of the protein have the capacity to induce the activation of the β-casein promoter (259). Typically, heterodimers of Stat5a and Stat5b are formed after their activation, but a relatively high concentration of Stat5a homodimers have also been detected in mammary epithelial cells (30). The predominant Stat5 dimer binds to the promoter sequence. The total levels of the Stat5 proteins do not vary much during mammary gland development, yet the kinetics of its tyrosine phosphorylation parallel the developmental profile of β-casein gene expression (162).

C/EBPβ in lactogenesis

Ccaat enhancer binding protein β (C/EBPβ) belongs to a family of transcription factors that is known to contain highly conserved, basic leucine zipper motifs at their carboxyl termini (bZIP). These bZIP motifs are responsible for the dimerization of the transcription factors and their DNA binding ability. Three C/EBPβ isoforms, LAP1/2 and LIP, are generated by a leaky ribosome-scanning mechanism (60). These isoforms are shown in **Figure 5**.

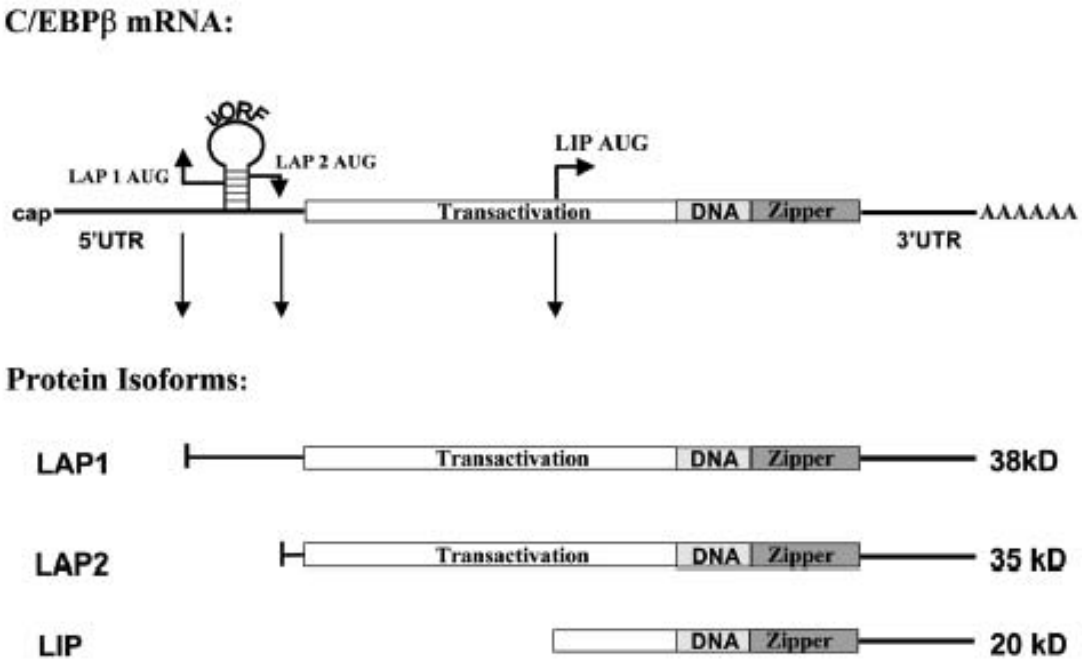
Two liver-enriched activating proteins (LAPs), are produced and these proteins share identical functionality. One liver-enriched inhibitory protein (LIP) is also produced, and this is a dominant negative transcription factor. The LAP-to-LIP ratio is the critical factor in controlling gene expression rather than is the absolute level of C/EBPβ protein in the cell (60). The β-casein proximal promoter contains approximately four C/EBPβ binding sites, and mutational analysis revealed that these sites are critical for the activation of the β-casein promoter (65). In fact, the absence of C/EBPβ reduced β-casein gene expression by 85-100% in the epithelium of the mouse mammary gland (218).

The total mRNA levels of C/EBPβ increase during pregnancy, decrease slightly during lactation, and increase again after 24 to 48 hours of involution (89). LIP displays a higher DNA binding affinity than do the LAP proteins, yet the enhancement of C/EBPβ transcription during lactogenic differentiation and thus the production of more LAP than LIP most likely results in the relief of transcriptional inhibition and allows the LAP of C/EBPβ to activate the β-casein promoter (211, 232).

Glucocorticoid Receptor (GR) and Yin Yang-1 (YY-1) in lactogenesis

Glucorticoids, synthesized in the adrenal cortex, are essential for the activation of the β-casein promoter. The promoter of the β-casein gene does not contain complete consensus glucocorticoid response elements (GREs), despite the importance of glucocorticoids in milk protein expression. Welte *et al*. determined by *in vitro* footprinting analysis that the promoter does contain half-GREs, which are sufficient for the binding of the GR to the β-casein promoter (274).

Figure 6. Isoforms of C/EBPβ. *Adapted from Breast Cancer Res 2002, **4**:113-121.*



In fact, the mutation or deletion of these sites severely diminishes or abolishes the cooperative effects of glucocorticoids and prolactin at the β-casein promoter in HC11 mouse mammary epithelial cells (151).

Yin Yang-1, or YY-1, is a ubiquitously expressed protein that can either activate or repress transcription. It acts as a repressor of β-casein gene expression in the absence of lactogenic hormones (170). YY-1 contains a domain responsible for the transcriptional activation functions of the protein at the N-terminus, while the C-terminus contains one of two repression domains. The β-casein promoter contains a negative regulatory region between nucleotides -150 and -110 (211, 228). The binding of YY-1 to this region has little effect following hormonal induction (170), but any mutation of this site leads to a stronger lactation-associated activation complex in the lactating mammary gland (211).

The role of chromatin structure in milk protein gene expression

The activation and inactivation of gene transcription may be brought about by modifications to the proteins in the bound transcriptional machinery or by interactions between the machinery and additional transcriptional activators. Transcriptional activation of genes is facilitated by the targeting of histone acetyltransferases to the genes, generating an open conformation at the promoter (185). Efficient binding of factors to hormone responsive elements of a promoter requires the recruitment of ATP-dependent complexes, such as the SWI complex (292). For review on chromatin structure at the β-casein promoter, refer to **Figure 3**. These complexes use the energy of ATP hydrolysis to make response elements accessible for the binding of the cognate

factors, which act as nucleation points for the recruitment of the basal transcriptional machinery (262). Alterations in the structure or composition of the nuclear matrix may reposition histone acetyltransferases and/or deacetylases, which are known to be bound to the nuclear scaffolding (54). It is also possible that the extracellular matrix induces or modifies cofactors which themselves have acetyltransferase or deacetylase activity (291).

Myers *et al*. found that the extracellular matrix (ECM) induces a complex interaction between bound transcription factors, the basal transcriptional machinery, and a chromosomally integrated template responsive to the acetylation state of the histones at the β-casein enhancer and promoter regions (185). There is evidence suggesting that the ECM maintains a high level of histone H4 acetylation upstream of the αs1-casein gene, especially at the level of a distal prolactin and ECM-sensitive enhancer region (123). Recently, Xu *et al*. investigated whether the ECM induces factors responsible for modulating the state of histones, and it was found that extracellular matrix molecules cooperate with prolactin to induce histone acetylation and the binding of transcription factors as well as the ATP-dependent SWI chromatin remodeling complex to the β-casein promoter (287). This finding suggests that transcription of the β-casein gene requires the cooperative action of the ECM, prolactin, chromatin remodeling factors and transcription factors.

Cooperative actions of prolactin and glucocorticoids in lactogenesis

Prolactin and glucocorticoids act by kinetically distinct mechanisms in mammary epithelial cells (64), yet the cooperation of both hormones in the production of milk proteins has also been well established (125). In HC11 mouse mammary epithelial cells,

treatment with hydrocortisone induces little expression of β-casein, while the addition of prolactin leads to a marked increase in expression (151). Importantly, the interaction between Stat5 and GR may be necessary for both the binding of GR to the half-GREs (243), as well as for the protection of Stat5 from dephosphorylation and deactivation by phosphatases (283). Also, pretreatment of HC11 mouse mammary epithelial cells with hydrocortisone leads to an increase in the LAP-to-LIP ratio of C/EBPβ (212), which may facilitate the prolactin-mediated relief of repression of β-casein transcription (228). The recruitment of p300/CBP, which is a coactivator with histone acetylase activity, to the promoter is another mechanism by which prolactin and GR synergistically regulate β-casein transcription (119).

The role of insulin in lactogenesis

Also important to β-casein expression is insulin. *In vivo*, insulin in combination with glucocorticoids works to maintain the alveolar structures of the mammary gland (216). During development, insulin has the ability to stimulate the growth of the mammary epithelium by increasing the progression through the cell cycle and decreasing apoptosis (171). One way in which insulin contributes to the activation of β-casein gene expression is by stimulating DNA polymerase activity (165). Also, the transcription factor C/EBPβ is known to be insulin-responsive (168). Unfortunately, the capability for C/EBPβ to mediate the effects of insulin on β-casein gene expression is untested. Since the insulin receptor is known to have the ability to stimulate the phosphorylation of Jak2, modulation of the Jak2/Stat5 pathway is another mechanism that has been suggested as a method of insulin-mediated activation of the transcription of β-casein (97).

The role of cell:matrix interactions during lactogenesis

As mentioned previously, the interaction between mammary epithelial cells (MECs) and the basement membrane (BM) is critical for successful lactogenic differentiation. The BM is established from the secretion of specific ECM components from the epithelial cells, and the subsequent assembly of a 100 nm thick matrix at the basal surface of the epithelium. This process is stimulated by the interaction of the cells with the matrix and stromal mediator proteins (130). While MECs adhere to the BM via various types of ECM receptors, the primary class of receptors is composed of heterodimeric α- and β-chain integrins (251). Other receptors whose specific roles remain undefined include dystroglycan, syndecan, and galactosyl transferase (245). In MECs stimulated to undergo lactogenic differentiation, signals from the matrix are mediated through integrins, since a function-blocking anti-integrin antibody severely diminishes the ability of cells to synthesize β-casein (246). As laminin is the primary component of the BM responsible for the activation of milk protein production, a study was done to determine the specific laminin receptor(s) that mediate the lactogenesis-specific responses. The study by Muschler *et al.* (183) determined that signals from laminin for β-casein transcription are inhibited in the presence of function-blocking antibodies against both the α6 and β1 integrin subunits. While it has been well established that integrin dimers containing the β1 subunit are required for proper gland development and epithelial cell differentiation (137, 188), it is not clear whether other αβ integrin complexes deliver key intracellular responses in this particular process.

Integrins bind ECM proteins when in an extended active conformation, and this binding promotes the formation of multi-protein adhesion complexes at the membrane

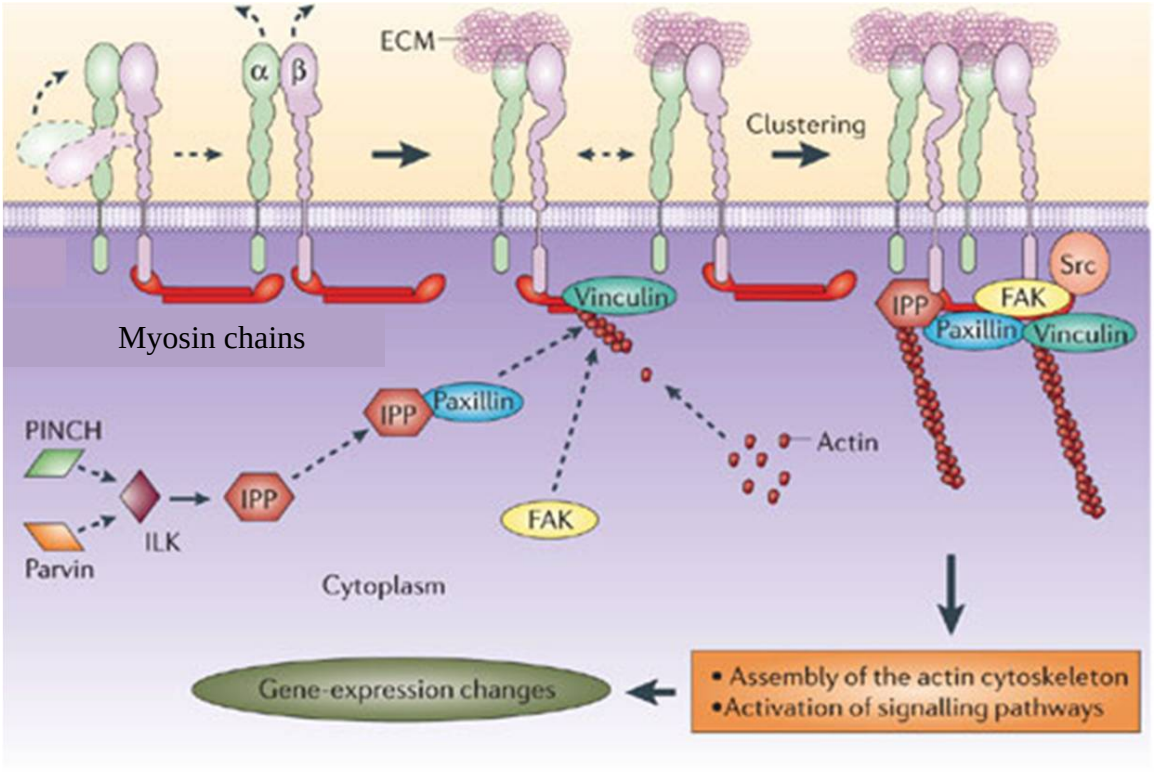
(88), including a variety of structural (i.e., vinculin), adaptor (i.e., paxillin, p130cas, parvin), and enzymatic proteins (i.e., focal adhesion kinase (FAK), integrin-linked kinase (ILK), Src) (28). The formation of these multi-protein complexes results in integrin clustering at the cell surface, and this clustering is referred to as a focal adhesion, which is depicted in **Figure 6**. Upon integrin activation, downstream signals primarily involve p130cas, paxillin, ILK, and Src (28). The complex is formed downstream of the adhesion-induced phosphorylation of FAK-Y397. Recruitment of Src causes tyrosine phosphorylation of additional sites on FAK, and the subsequent FAK-mediated phosphorylation of p130cas and paxillin (226). Src has been shown to be involved with both MEC differentiation and proliferation through its effects on ERα and p130cas (134). FAK activation may also contribute to cell survival by direct activation of the phosphatidylinositol 3- kinase (PI3K)/Akt pathway (19).

The role of the cell:matrix interactions on MEC survival and proliferation

Detachment from the ECM leads to a type of apoptosis of epithelial cells called anoikis (90), thus one of the roles most commonly ascribed to integrin-mediated adhesion is cell survival. Mammary-specific dominant-negative β1 integrin has been shown to result in a decrease in MEC proliferation and increased apoptosis during pregnancy and lactation (74). In MECs, overexpression of FAK can prevent anoikis (91). In a study using FAK mammary conditional knockout mice, it was determined that the knockout females display severe lobulo-alveolar hypoplasia and secretory immaturity during pregnancy and lactation (186). The cells of these glands also showed a decrease in phosphorylated Erk1/2, cyclin D1, phosphorylated Stat5, and diminished proliferative

capacity (186). Also, cells that lack a functional β1 integrin display enhanced apoptosis in *in vitro* culture (156).

Figure 7. Formation of focal adhesion complexes. *Adapted from Legate et al*., (152).



Laminin-1 (LM-1) is the stromal component that plays the largest role in the integrin-mediated survival pathway. It has been shown to suppress anoikis more efficiently than other ECM proteins such as collagen I (210). Moreover, the cell:BM interaction is commonly viewed as a positive checkpoint to suppress apoptosis, ensuring that MECs are only maintained within ducts and alveoli (209).

Upon the genetic deletion of β1 integrin, MECs undergo cell cycle arrest and glands display defective development *in vivo*, suggesting that mammary epithelial cell proliferation requires integrin-mediated ECM adhesion (156). Typically, cells proliferate in response to hormonal stimulation only if the adhesion context is permissive. Moreover, cells derived from pregnant mice do not proliferate in response to estrogen or progestin treatment, suggesting that the ECM assists the MECs to respond in accordance with their developmental stage (286). In addition, there is evidence indicating that a hormonal signal from insulin is necessary to suppress mammary cell apoptosis, indicating that BM proteins cooperate with soluble factors in survival signaling (76).

Signals generated as a result of the binding of growth factors to their receptors also rely on complexes formed through the interaction of the cells with matrix proteins and stromal mediators. The receptors for most growth factors are of the receptor tyrosine kinase family, which is known to display cross-talk with integrin signaling complexes (98). It has been shown that the adhesion of epithelial cells to LM-1 reduces the binding of epidermal growth factor (EGF) to its receptors, resulting in a decrease in the EGF-dependent phosphorylation and activation of Erk1/2 (280). Mammary epithelial cells cultured in two dimensional environments on stromal ECM display different

responsiveness to EGF signaling compared with those grown in three dimensional matrices on purified BM (184).

The role of cell:matrix interactions on milk protein production

As previously stated, the loss of β1 integrin function contributes to defective milk protein production in mammary epithelial cells, though the exact mechanism involved remains unclear. It is known that mice genetically lacking functional β1 integrin display alveolar structures with disrupted morphology and the absence of Stat5 nuclear translocation in response to prolactin stimulation (156, 188). A study of the mechanism of β1 integrin *in vivo* by Faraldo *et al*. (75) determined that distruption of β1 integrin function induced precocious dedifferentiation of the secretory epithelium in the mammary gland. This was shown by a premature decrease in β-casein and WAP mRNA levels, accompanied by the inactivation of Stat5 and an upregulation of NF-κB (75). Another study has shown that the DNA binding activity of Stat5 was present only in extracts of mammary cells that had been cultured on BM, indicating that the activation state of Stat5 is at least partially regulated by the ECM (247).

It has been suggested that the link between β1 integrin signaling and prolactin-induced signaling is via the integrator of adhesion signaling, Rac1 (3). It was suggested by Akhtar and Streuli (3) that integrin-containing adhesion complexes and Rac1 cooperate together to allow prolactin-mediated Stat5 nuclear translocation and the resulting transcription of milk proteins, but a definite link has not yet been determined. The question of how the integrin-mediated signals result in proper terminal differentiation and milk protein production still remains unanswered.

Connective Tissue Growth Factor

Using microarray analysis to investigate factors involved in the process of lactogenesis, our lab found that Connective Tissue Growth Factor (CTGF/CCN2) is one of the most highly upregulated proteins in HC11 mouse mammary epithelial cells that had been stimulated to undergo lactogenic differentiation (271).

CCN family of matrix-associated proteins

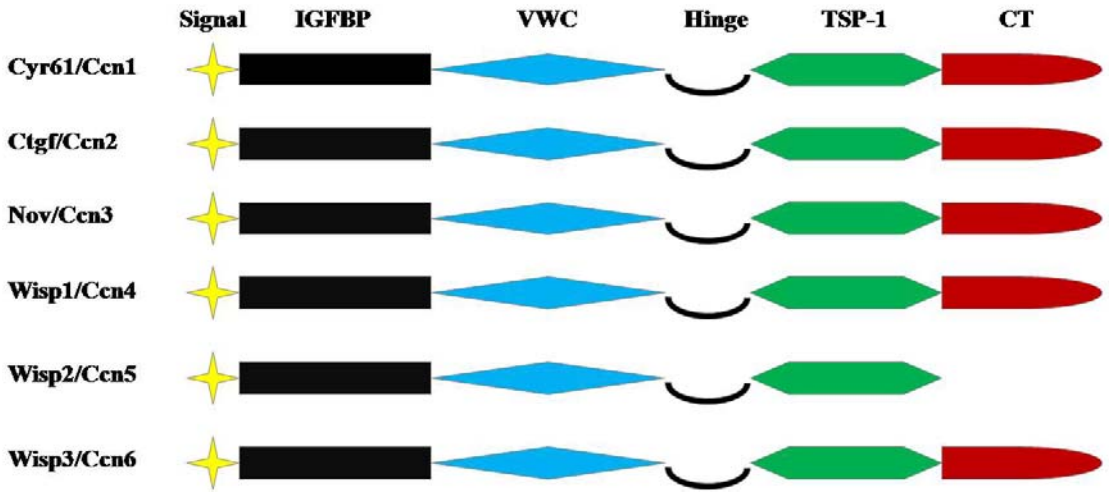
The CCN family of matrix associated proteins is comprised of six cysteine-rich proteins and is named for the first three proteins of the family to be discovered. The family includes the proteins CYR61/CCN1, CTGF/CCN2, NOV/CCN3, and the Wnt-induced secreted proteins 1-3 (Wisp) Wisp1/Elm1/CCN4, Wisp2/Rcop1/CCN5, and Wisp3/CCN6.

The six members of this family share a modular structure of an N-terminal signal peptide followed by four homology domains (202), as shown in **Figure 7**. Domain 1 bears sequence homology to IGF binding proteins (IGFBP), domain 2 is homologous to a von Willebrand type C repeat (vWC), domain 3 resembles thrombospondin 1 (TSP-1), and domain 4, the carboxyl-terminal (CT) domain, contains a cysteine knot motif (18, 149). Tissue-specific CCN isoforms, derived from post-translational processing, proteolytic cleavage between the distinct modules or alternate splicing, have been detected (202).

This family of proteins is the product of immediate-early genes, and its members are secreted into the extracellular environment where they can associate with the cell surface and ECM components (254). Early studies of CCN proteins advanced down two

main paths: the CCN proteins as growth factors (20) and the CCN proteins as ECM-associated cell adhesion molecules (135). Currently, the characterization of CCN proteins has shifted in support of the latter view as the majority of researchers now define CCN proteins as matricellular effectors.

Figure 8. The CCN family of matrix-associated proteins.



The members of the CCN family were first identified as secreted proteins induced by mitogenic growth factors or oncogenes (21). The CCN family of proteins displays tremendous bioactive diversity, and the molecular signals that induce this diversity are believed to reside within the modular structure-function relationships and bioavailability of each family member. The characterization of Wisp2/CCN5, which lacks the CT domain, was an early indicator that CCN proteins expressed under normal conditions may display critical structural differences (202). The multidomain structure of the CCN proteins allows them to interact with several other proteins whose bioavailability may differ among tissues and with developmental stages (203). For example, in activated rat hepatic stellate cells, the TSP-1 domain of CTGF/CCN2 binds to low-density-lipoprotein receptor-related protein (LRP) (86). In various cell types, the CT domain of CTGF/CCN2 binds heparin (9), while the CT domain of CYR61/CCN1 binds the $\alpha_m\beta 2$ integrin complex in monocytes (230), and the CT domain of NOV3/CCN3 binds fibulin 1 and Notch 1 (204). The detection of 10-20 kDa CTGF/CCN2 proteins in pig uterine luminal flushings was the first indication that bioactive variants of this CCN family member are generated through proteolysis of the 38 kDa CTGF/CCN2 primary translational product (10). Moreover, a variant of CTGF/CCN2 lacking the CT domain was isolated in high concentration in primary human osteoblasts (203), supporting the idea that different combinations may be the key to the flexible functionality of the CCN proteins.

The individual proteins of the CCN family exhibit diverse functions regulating many important biological processes including cell attachment (7, 34, 111), migration (96, 158), cell survival (7, 153, 159), growth (1, 118), and differentiation (21, 178).

Specifically, CCN1-3 have been shown to promote angiogenic activity by supporting cell adhesion, stimulating cell migration, enhancing the survival of endothelial cells and promoting endothelial tubule formation (7, 159, 176, 177). Highlighting the diversity of function, during skeletal development CCN1 and 2 promote chondrogenesis and the differentiation of chondrocytes, while CCN3 inhibits osteogenic differentiation (1, 118, 146). In certain forms of cancer, particularly breast cancer, CCN1 and 2 have been shown to promote angiogenesis, enhance the growth and survival of cancer cells as well as their resistance to apoptosis, while CCN4 and 5 have been shown to inhibit tumorigenicity (59, 66, 257). The diverse functions of the CCN family members have been well reviewed (35). This study will focus on the function of CTGF/CCN2.

CTGF/CCN2 gene and protein expression

CTGF/CCN2 was first isolated from human umbilical vein endothelial cell (HUVEC) culture supernatants using an antibody against platelet-derived growth factor (PDGF) (20). During development, CTGF/CCN2 is robustly expressed in the mesenchyme, including hypertrophic chondrocytes in the growth plate of cartilage (288). During embryonic development, the lack of CTGF/CCN2 results in a failure of the embryo to induce the expression of bone specific matrix proteins such as aggrecan, as well as a failure of chondrocyte proliferation (118). This results in the overall inability of the rib cage to ossify properly and, if born, mice with a homozygous deletion for CTGF/CCN2 die soon after birth. The loss of the CTGF/CCN2 has also been associated with defective angiogenesis as CTGF/CCN2 is known to regulate levels of vascular endothelial growth factor (VEGF) (118). The CTGF/CCN2 knockout model provides

evidence that CTGF/CCN2 is a key regulator, coupling ECM remodeling to chondrogenesis, angiogenesis and the growth plate. In the adult, the 2.4kb CTGF/CCN2 transcript is most commonly expressed in the heart, brain, lung, liver, muscle, kidney, and pancreas, with the greatest levels of expression in the kidney (196).

As mentioned previously, the CTGF/CCN2 protein is secreted and can be cleaved into functional variants. The hinge region between domains 2 and 3, which separates the N-terminal region from the C-terminal region of CTGF/CCN2, can be cleaved by most proteases including MMPs 1, 2, 3, 7, 9, and 13, as well as elastase and plasmin (99). The latter proteins also have the ability to cleave the regions between individual domains in either the N-terminal portion or the C-terminal portion. As is true for most of the CCN family members, *in vitro* studies have suggested that the diverse actions of CTGF/CCN2 may result from the relative amounts of full-length and clipped product (68). Individually, each domain has a role in the functionality of CTGF/CCN2. It is important to note that although domain 1 of CTGF/CCN2 bears homology to IGF binding proteins, this domain cannot functionally replace that of bona fide IGF binding proteins (289). The vWC domain has been implicated as a binding site for one of the most common regulators of CTGF/CCN2, transforming growth factor β (TGFβ) (1). There is evidence that the TSP-1 domain binds to sulfated glycoconjugates (61), while the CT domain is responsible for the dimerization of the protein as well as its ability to bind to the cell surface through heparin sulfate-containing proteoglycans (182).

CTGF/CCN2 is a secreted protein and is involved in many cellular processes including cell growth and proliferation, differentiation, angiogenesis, adhesion and chondrogenesis (95, 149, 187). It has been recognized as an important factor in fibrotic

processes and wound healing, as well as in certain forms of cancer, though its molecular mechanism in each condition has yet to be fully understood.

Regulation of CTGF/CCN2

While regulated by a number of other non-nuclear factors including glucocorticoids and Wnt-induced factors, CTGF/CCN2 is most widely regarded as a downstream mediator of transforming growth factor β (TGFβ).

In adults, CTGF/CCN2 is induced during wound healing and is overexpressed in fibrosis (116). In a recent study by Dziadzio *et al.* (68), the N-terminal region of CTGF/CCN2 was detected in the blister fluid of scleroderma patients and is now considered an important biomarker for the severity of skin fibrosis. A major component of the fibrotic process is the over-activation of TGFβ. TGFβ is known to induce CTGF/CCN2 through a complex network of transcriptional interactions requiring Smads, protein kinase C (PKC) and Ras/MAPK signaling, as well as the Ets-1 transcription enhancer factor element in the CTGF/CCN2 promoter (39, 150, 261). An *in vivo* model of wound repair provides evidence that TGFβ and CTGF/CCN2 are overexpressed in a coordinated fashion (116).

It has been suggested that CTGF/CCN2 acts as a downstream mediator of TGFβ to enhance scar tissue formation (178). It has also been shown that fibroblasts involved in keloid formation have an intrinsic upregulation of CTGF/CCN2 and display an exaggerated competence for CTGF/CCN2 transcription in response to stimulation with TGFβ (45). Together, these results suggest that the suppression of CTGF/CCN2 may reduce a progressive fibrotic response and pathologic scar formation. A study by Black

et al. (14) provided evidence that the Rho family of GTPases, Rac1 and cdc42, are the principal mediators of the TGFβ-stimulated expression of CTGF/CCN2 in primary human gingival fibroblasts. The regulation of CTGF/CCN2 in gingival fibroblasts may therefore also provide therapeutic opportunities to treat fibrotic diseases.

As mentioned previously, CTGF/CCN2 is highly expressed in the adult kidney. Moreover, CTGF/CCN2 is upregulated in many diseases of the kidney and has been shown to contribute to renal fibrosis and tubuloepithelial transdifferentiation downstream of the actions of TGFβ (221). CTGF/CCN2 expression in the kidney also enhances various ECM proteins previously implicated as a result of TGFβ upregulation in diabetes-associated kidney disease (42). However, CTGF/CCN2 is also expressed in embryonic and fibrotic fibroblasts independently of TGFβ, suggesting that other factors may also mediate its expression (41, 109). In fibrotic and wound healing conditions, other positive regulators of CTGF/CCN2 include endothelin-1, thrombin, glucose, and angiotensin II (32, 79, 181), while negative regulators include TNFα, cAMP, and prostacyclins/prostaglandins.

CTGF/CCN2 can also be induced by stress factors including high glucose, mechanical load, or hypoxia (141). A *cis*-element in the 3' untranslated region of CTGF/CCN2 that contains a conserved sequence has been discovered that, when bound to an unknown stress-induced nuclear factor, regulates expression via control of mRNA stability (140, 145). Multiple studies have also identified CTGF/CCN2 as a potential target of Wnt signaling. Labbe *et al*. (147) found CTGF/CCN2 to be one of nine genes upregulated in two separate models of Wnt-induced tumors in mice. This upregulation of CTGF/CCN2 was enhanced when the mice were treated with TGFβ, suggesting that

TGFβ and Wnt may act cooperatively to induce the transcription of CTGF/CCN2. In osteoblasts, it was found that CTGF/CCN2 can be regulated by Wnt5a, and this regulation is β-catenin-dependent (167). Eguchi *et al*. (70) found that human nuclear MMP3 can act as a trans-regulator of CTGF/CCN2 in chondrocytic cells by the activation of the promoter region. In the chondrocytic cell line HCS-2/8, CTGF/CCN2 is also transcriptionally induced by dexamethasone via a dexamethasone response element in its promoter (145). Together this group of data highlights the diverse mechanisms possible in the regulation of the expression of CTGF/CCN2 in various cell types and conditions.

Biological functions of CTGF/CCN2

As previously mentioned, CTGF/CCN2 is involved in a diverse set of cellular functions. In concert with other growth factors, CTGF/CCN2 has been shown to promote the proliferation of many cell types, matrix production, angiogenesis, chondrogenesis, and the formation of granulation tissue (20, 22, 80). Directly, CTGF/CCN2 promotes cell adhesion and migration in a variety of cell types (7, 48, 135). The specific details of a relevant selection of these functions are discussed below.

CTGF/CCN2 activity in chondrogenesis and chondrocytic differentiation

It was recently determined that the exogenous expression of CTGF/CCN2 promotes cell migration and the recruitment of mesenchymal stem cells (167). The same study found that CTGF/CCN2 is upregulated during the early stage of osteogenic differentiation, but is subsequently downregulated as the differentiation potential of committed pre-osteoblasts increases. The expression of CCN family members,

CTGF/CCN2 in particular, in proliferating and differentiating human chondrocytes was also analyzed by Schutze *et al*. (231). It was determined by this study that CTGF/CCN2 is differentially expressed in the chondrocytes during the different phases. Together, these results suggest that CTGF/CCN2 may be a critical factor for bone marrow-derived mesenchymal stem cells in the management of proliferation and the initiation of specific differentiation pathways. *ctgf/ccn2* $^{-/-}$ chondrocytes showed a decrease in type II collagen and aggrecan, concomitant with impaired DNA synthesis and reduced adhesion to fibronectin (192). These reductions were associated with decreased levels of α5 integrin, focal adhesion kinase (FAK), and Erk1/2 phosphorylation. The mechanistic findings from the study by Nishida *et al*. suggest that CTGF/CCN2 exerts its effects in chondrocytes through its ability to regulate ECM production and integrin expression, to engage integrins, and to activate integrin-mediated signaling pathways (192).

The role of CTGF/CCN2 in cell proliferation

In cooperation with other factors, CTGF/CCN2 is known to enhance proliferation in a dose-dependent manner in various cell types, including but not limited to osteoblasts and chondrocytes (187, 194), choroidal endothelial cells (101), smooth muscle cells and articular cartilage cells (72, 193), periodontal ligament-derived cells (6), and hepatic stellate cells (199). The exact mechanism by which CTGF/CCN2 contributes to proliferation in these cell types is not yet fully understood, but it has been suggested that the outcome is due to the ability of the protein to induce activation of the mitogenic factors Mek1/2 and Erk1/2 (48, 85, 264, 266), as well as the activation of p90RSK, ATF2, and Akt (266).

The role of CTGF/CCN2 in cell adhesion

One of the most studied functions of CTGF/CCN2 is its role in cell adhesion. It performs many of its adhesion-related functions through interaction with integrin complexes, as well as through the interaction with heparin sulfate proteoglycans (HSPGs) and the LDL-related protein (LRP) (9, 34, 233). The specific complexes through which CTGF/CCN2 exerts its effects differ depending on cell type (40, 229). For instance, it is known that CTGF/CCN2 contributes to the adhesion of human foreskin fibroblasts through α6β1 integrin, of endothelial cells through $\alpha_v\beta3$, of blood monocytes through $\alpha_m\beta2$, and of platelets through $\alpha_2\beta3$ integrin complexes (7, 34, 40, 229). In each system, the exact signaling mechanisms by which CTGF/CCN2, or proteolytic variants of CTGF/CCN2, exerts its effects on adhesion remain areas of investigation.

As stated previously, the full length and cleaved variants of CTGF/CCN2 may display differing functions and each may act through diverse mechanisms in specific tissues and under certain conditions. One reason for this discrepancy may be due to the location of the particular integrin complex binding site within the domain structures. In cell types where CTGF/CCN2 is said to bind to and interact with $\alpha_5\beta1$ integrin, such as in pancreatic stellate cells (87), mesangial cells (276) and chondrocytes (111), the binding site has been determined to be in the CT domain, or domain 4 of CTGF/CCN2. In many cell systems, CTGF/CCN2 has been found to enhance binding of the cells to the ECM protein fibronectin (16, 80, 276). Integrin $\alpha_5\beta1$ is a common receptor for fibronectin in many cell types. In human mesangial cells, treatment with CTGF/CCN2 enhanced the levels of β1 integrin on the surface of the cells (276). Together, these investigations have

provided evidence suggesting that the CT domain of CTGF/CCN2 acts primarily through the β-integrin subunit of the $\alpha_5\beta1$ complex to enhance the adhesion of cells to fibronectin.

CTGF/CCN2 has been shown to enhance the adhesion of activated monocytes via an interaction with the $\alpha_m\beta2$ integrin complex (229, 230). The binding site for the $\alpha_m\beta2$ was found to be located within the CT domain, or domain 4, of CTGF/CCN2 (230), and this interaction is specific for the α_mI subunit of the complex. It has also been demonstrated that vascular endothelial cell and tubular epithelial cell adhesion is mediated through the interaction of CTGF/CCN2 with the $\alpha_v\beta3$ integrin complex (120), and the binding site for this complex is also located within the CT domain, or domain 4, of CTGF/CCN2 (236).

The TSP-1 domain, or domain 3, of CTGF/CCN2 has been demonstrated to contain the binding site for the α6β1 integrin complex (154). The interaction between CTGF/CCN2 and the α6β1 integrin complex enhances the adhesion of skin fibroblasts (34, 154), as well as the adhesion of hepatic stellate cells (255) to laminin, which is the most common ligand for the α6β1 complex. In order for cells to adhere to the ECM properly, they form focal adhesions, which were described previously. A study by Chen *et al*. (34) found that CTGF/CCN2 induces extensive formation of α6β1 integrin complex-containing focal adhesions in human skin fibroblasts, as well as the subsequent enhancement of signaling through the focal adhesion complex of FAK, paxillin, and Rac1. The same study also provided evidence that CTGF/CCN2 induces activation of Erk1/2 as well as MMPs 1 and 3.

The role of CTGF/CCN2 in cell growth and survival

Based on the idea that CTGF/CCN2 is an important mediator of cell adhesion in many cell types, investigation into its role in growth and survival has been warranted. Typically, in order to survive, cells must adhere to the ECM. This occurs predominantly through the interaction of cellular integrins with matrix proteins. This interaction stimulates signal transduction pathways that lead to the activation of cell growth and survival factors, such as Erk1/2, Akt, and Bcl-2. Should the cells lose their ability to adhere to the matrix, they lose their survival signaling mechanisms and apoptotic signaling mechanisms are initiated during a process referred to as anoikis. The details of this mechanism have been discussed previously. The role of CTGF/CCN2 in cell growth and survival differs between cell types as it has been shown both to enhance survival (105, 255, 264), as well as enhance apoptosis (106, 160).

A study using embryonic fibroblasts isolated from CTGF/CCN2-deficient mice determined that CTGF/CCN2 is a necessary cofactor of TGFβ for cell:matrix adhesion, subsequent activation of the FAK/PI3K/Akt signaling cascade, and for induction of FAK/Akt-dependent genes (235). In rat neonatal cardiac myocytes, CTGF/CCN2 stimulation, particularly with the CT domain, enhanced the cell surface area and amino acid uptake (100), which correlated with enhanced adhesion and survival of the cells. The same study also demonstrated that treatment of the cardiac myocytes with CTGF/CCN2 resulted in the activation of growth and survival factors Erk1/2 and Akt, as well as p38MAPK and JNK (100). Treatment of human mesangial cells with CTGF/CCN2 enhanced their survival through the induction of MAP kinase phosphatase-1(Mkp-1) and subsequent stabilization of the anti-apoptotic protein Bcl-2 (264). In

survival signaling pathways, it is the job of Mkp-1 to dephosphorylate MAP kinases p38 and JNK, allowing for accumulation of the anti-apoptotic protein Bcl-2.

In human renal epithelial cells (164), rat aortic smooth muscle cells (72), normal rat kidney fibroblasts (142), and human mesangial cells (105), CTGF/CCN2 was shown to dose-dependently enhance viability by the activation of growth and survival factors Erk1/2 and Akt, as well as the suppression of apoptotic factors such as caspase 3 (72). In contrast, CTGF/CCN2 may induce apoptosis in renal pericytes (160) and MCF7 human breast cancer cells (107). While it is difficult to determine whether apoptosis is a physiologically relevant function of the protein due to the fact that these cells are typically treated with very high concentrations, CTGF/CCN2 is known to accumulate in the local extracellular environment, thus the possibility cannot be excluded (93). The current body of evidence that CTGF/CCN2 supports survival, compared with that which supports apoptosis, favors the activity of CTGF/CCN2 as being an anti-apoptotic factor.

CTGF/CCN2 in cancer

Recently, evidence has been provided showing that CTGF/CCN2 is elevated in a number of cancers, including pancreatic cancer (275), breast cancer (285), glioblastoma (198), esophageal (139), melanoma (144), acute lymphoblastic leukemia (263), and hepatocellular carcinoma (293). These studies implicate CTGF/CCN2 in both the development and progression of the disease. It has also been suggested that CTGF/CCN2 is likely to be one of the key regulators of angiogenesis in the tumor-reactive stromal microenvironment (290). In a pancreatic cancer system, Dornhofer *et al*. (66) showed that CTGF/CCN2 promotes anchorage-independent growth both *in vitro* as well as tumor

xenograft growth *in vivo*. As is true with non-transformed systems, the exact function and mechanism of CTGF/CCN2 varies with the cell type and condition and is still a matter of investigation.

CTGF/CCN2 and other CCN members in tumorigenic and non-tumorigenic mammary glands

CYR61/CCN1 has been shown by various studies to be highly expressed in tumors of the mammary gland (122, 224, 257, 285). Elevation of this protein in breast cancer is typically associated with nodal involvement and metastasis (122), which are both associated with poor patient prognosis. CYR61/CCN1 has been shown to be regulated by 17β-estradiol (224), and its angiogenic properties may be necessary for the growth of mammary adenocarcinomas (223). In some of these same studies, low levels of CTGF/CCN2 were associated with similar outcomes (122). In others, CTGF/CCN2 was shown to be elevated in tumors of the mammary gland (285) as well as in osteolytic bone metastases of breast cancer (127).

CTGF/CCN2 is elevated in advanced stages of breast cancer, yet its exact function is still under investigation (37, 237). A study by Chen *et al*. (37) found that the overexpression of CTGF/CCN2 in MCF7 human breast cancer cells resulted in enhanced migration and focal adhesion-complex aggregation. The same study also found that the inhibition of the $\alpha_v\beta3$ integrin complex attenuated CTGF/CCN2-mediated activation of FAK-Erk1/2-S100A4 signaling and subsequently the ability of the cells to migrate, suggesting that the mechanism of CTGF/CCN2 in advanced breast cancer is related to its ability to enhance the adhesion signaling of epithelial cells. Another study applied a

quantitative proteomic approach to identify proteins whose levels are altered in a mouse model of breast cancer and found that CTGF/CCN2 was increased in the plasma of mice displaying mammary tumors of ~1.0 cm or larger (207).

It is widely accepted that CTGF/CCN2 has the ability to enhance the adhesion and angiogenic properties of tumorigenic cells, yet one function of CTGF/CCN2 that is under current investigation is its role in osteolytic metastasis. Together, chondrocytic HCS-2/8 and MDA-MB-231 metastatic breast cancer cells produce over six times more CTGF/CCN2 than any other cell type (69). A study by Shimo *et al*. (237) investigated whether, or how, the neutralization of CTGF/CCN2 prevented bone metastasis. In this study, MDA-MB-231 cells treated with a CTGF/CCN2 neutralizing antibody displayed a decrease in osteolytic bone metastasis and microvasculature when injected into a mouse model.

In a pituitary cell model, which relates to the mammary gland via regulatory hormones produced by the pituitary, CTGF/CCN2 was found to be one of six genes that showed elevated expression in cell monolayers (71). Other upregulated genes included osteopontin and α_v integrin. With further investigation into pituitary-mediated enhancement of CTGF/CCN2, elevated CTGF/CCN2 transcripts were detected in pregnant rats and also in rats treated with dexamethasone (71). Together with the role of CTGF/CCN2 in breast cancer phenotypes, this data suggests that CTGF/CCN2 plays an important, yet relatively unexplored role in the cells of the mammary gland.

Chapter 2: Experimental Methods

Cell Culture and Lactogenic Hormone-induced Differentiation

HC11 mouse mammary epithelial cells, provided by Dr. Nancy Hynes, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals), 5μg/ml insulin, 10mM HEPES and 10ng/ml epidermal growth factor (EGF) as described (115, 169). Prior to stimulation with lactogenic hormones, HC11 cells were grown to confluence and maintained for 3-5 days to establish competence (11, 252). The cells were then grown in media without EGF for 24 hours. To induce lactogenic hormone-induced differentiation, the cells were rinsed twice with 1X PBS and then incubated in differentiation media, which is RPMI with a combination of dexamethasone (0.1μM), 5μg/ml insulin, and 10μg/ml prolactin, herein referred to as DIP. The cells were harvested at the stated times after the addition of DIP. Lactogenic differentiation was also characterized by the formation of domed structures referred to as mammospheres, which are visualized by phase contrast microscopy and manually enumerated.

Immunohistochemistry of Mouse Mammary Tissue

Tissues from whole mounts of FVB mouse mammary glands were paraffin-embedded and sectioned (American HistoLabs). The anti-CTGF/CCN2 primary antibody (Santra Cruz Biotechnology) was prepared at a 1:100 dilution. Staining was performed using the following from Vector Labs: R.T.U. VectaStain Universal Quick Kit and DAB Substrate Kits. In brief, the procedure was as follows: the tissues were hydrated in

Citrisolv followed by a graded alcohol series, then blocked with 2% horse serum and incubated in 3% hydrogen peroxide to block endogenous peroxidase activity, followed by a wash in 1X PBS. The tissues were incubated with the diluted primary antibody overnight at 4°C and the biotinylated pan-specific secondary antibody (~200µl/tissue) was added for 5-10 minutes at room temperature. The primary antibody was tested against other anti-CTGF/CCN2 antibodies and was determined to be the best in terms of detecting the 38kd protein. The tissues were washed in PBS, followed by a 5 minute incubation with the strepavidin-peroxidase complex solution. The slides were then washed in PBS for 5 minutes followed by the addition of the DAB solution, and the reaction was stopped by immersing the slides in hematoxylin for counterstain. The tissues were dehydrated; the coverslips were mounted with permount and dried overnight prior to visualization with the microscope.

Primary Mammary Epithelial Cell Culture Preparation

Primary mammary epithelial cell cultures were prepared from pregnant FVB/N mice as previously reported (239, 271). The mammary glands were removed, minced, and treated with collagenase (Type F, Sigma) overnight. The organioids were recovered by centrifugation and plated on plastic tissue culture plates. Primary cultures were maintained in DMEM supplemented with 10% fetal bovine serum, 5µg/ml insulin and 10ng/ml EGF. After 3 days of attachment and growth, fibroblasts were removed by brief trypsin treatment and were maintained as separate cultures. Culture purity was determined by looking at the levels of vimentin and cytokeratin as markers for fibroblasts

and epithelial cells, respectively. Primary mammary epithelial cultures were induced to differentiate in an analogous way to HC11 cells.

Adenovirus Preparation and Infection

CTGF/CCN2 full length and variant viruses were prepared as described by Wang *et al*. (271). Viruses were amplified and high titer stocks were purified by CsCl gradiant centrifugation. HC11 and primary mammary epithelial cell cultures were infected with replication defective adenoviruses or control vectors encoding GFP in a low volume of virus-containing media at an MOI of 10-20 for 5-6 hours. Fresh growth media without virus was added to cells for 24 hours prior to additional treatment of the cells.

Construction of the CTGF Tet-off cell line

The HC11 cell line containing the pTet-Off plasmid (Clontech) has been described (31). This HC11-TRE line was infected with a pREV-TRE (Clontech) retroviral vector expressing a Tet-regulated HA-tagged open reading frame of CTGF. This vector is used to express HA-CTGF in response to the removal of doxycycline (Dox) as described previously (271). Briefly, the TRE contains seven direct repeats of the tetO operator sequence, which can be bound to tTA transactivators upstream of a minimal CMV promoter. There is a 5' viral LTR that regulates the expression of the transcript that contains the viral packaging signal and the hygromycin resistance gene. The TRE is an internal promoter. The HC11-TRE cell line was infected with pREV-TRE-CTGF retroviral vectors prepared by the transfection of the plasmid vector DNA into the PA317 packaging cell line. Following infection, the HC11-TRE-CTGF cell lines were selected in hygromycin (200µg/ml) and doxycycline (2µg/ml) for ten days.

Twenty-four colonies were isolated and expanded into cell lines. The clonal cell lines were tested for expression of vector encoded HA-CTGF mRNA and protein following the removal of doxycycline from the culture media. Consistantly, HC11-TRE vector control cells and HC11-TRE-CTGF cells were grown in the absence of doxycycline for 96 hours prior to use for experimentation.

RNA isolation, RT-PCR, Northern blotting, Southern blotting and Real-time PCR

Total RNA was isolated and purified using the TriPure reagent (Roche). Northern blots were prepared with 10μg of total RNA separated on a 1% agarose-formaldehyde gel and transferred to a nylon filter. Reverse transcriptase PCR (RT-PCR) was performed with isolated total RNA using the GeneAmp RNA PCR Kit and GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Primers for β-casein amplification were as follows: Fwd 5' CATCCTTTCAGCTTCACC, Rev 5' AGAGACAGCTGGGTCTGAG. Primers for mouse β-actin were as follows: Fwd 5' CTAAGGCCAACCGTGAAAAGA, Rev 5' GAGGTCTTTACGGATGTCAAC. PCR products were electrophoresed on a 1% agarose gel. The gel was either photographed and quantified by densitometry on a CCD camera (Fuji), or incubated with 1X denaturation buffer (1M NaCl, 0.5M NaOH) for 30 minutes, followed by a rinse with water and a 45 minute incubation with 1X neutralization buffer (0.5M Tris, 1M NaCl) for Southern blot analysis. DNA was transferred to a nylon filter in 10X SSC. Northern and Southern blots were hybridized as described previously (271)**.** Briefly, for northern blot analysis the nylon filter was UV cross-linked followed by a 2 hour incubation with hybridization buffer (10ml buffer/filter of 5ml deionized formamide, 2.5ml 20X SSPE, 1ml 10% SDS, 200μl 50X Denhardts

solution, 300µl of 10mg/ml salmon sperm DNA, 1ml water) at 42°C in a rotation chamber. The DNA probe was prepared using the Prime It II – Random Primer Labeling Kit (Strategene). Briefly, 100ng of DNA probe was incubated with 10µl of random primer, mixed with dCTP buffer and exo-klenow enzyme and finally ^{32}P radiolabeled. Probes were then incubated with salmon sperm DNA and unincorporated nucleotides were removed by running samples through Probe Quant spin columns (GE Healthcare). Filters were incubated with radiolabeled probes overnight followed by three 20min washes with wash buffer (0.1X SSC, 0.5% SDS). The probes for CTGF, β-casein and actin have been described (31, 271)**.** The probe used for detecting CTGF was a 1050bp fragment corresponding to the sequence from nucleotide 211 to nucleotide 1260 in mouse CTGF (accession number NM_010217). The probe used for detecting β-casein was a 601bp fragment corresponding to nucleotides 3-604 from the mouse β-casein (accession number X04490.1). Real-time PCR was performed using SYBR green PCR kits and a 7500 Real-Time PCR instrument (Applied Biosystems). The primers used for detecting mouse β-actin by real-time PCR include: Fwd 5' TTACTGCTCTGGCTCCTAGCAC 3', Rev 5' GACTCATCGTACTCCTGCTTGC 3'. Primers used for detecting mouse β-casein by real-time PCR include: Fwd 5' TCACCTCCTCTCTTGTCCTC, Rev 5' TGTTCAACAGATTCCTCACTG.

Cell Lysate Extraction and Western blotting

The procedures for western blotting have been described (31, 67, 82, 271). Briefly, cells were harvested and washed in 1X PBS. Cell pellets were lysed in an appropriate volume of RIPA buffer (1% NP40, 0.5% DOC, 0.1% SDS, 150mM NaCl,

5mM $MgCl_2$, 25mM HEPES) plus the protease inhibitors AEBSF (20μg/ml), aprotinin (5μg/ml), leupeptin (5μg/ml), β-glycerol phosphate (100μM), and sodium orthovanadate ($NaVaO_{4)}$ (1mM). Lysates were normalized for protein concentration using the BCA Protein Assay (Pierce) and subsequently electrophoresed on Tris-Glycine gels (Invitrogen), the percentage of which was determined depending on protein size. Proteins were transferred to PVDF membranes which were blocked with 5% non-fat milk and probed with relevant antibodies. The antibodies used in this study include: mouse anti-β-actin (Sigma), goat anti-CTGF (Santa Cruz Biotechnology), mouse anti-β1 integrin (BD Transduction), mouse anti-FAK (BD Transduction), rabbit anti-phospho-FAK (Upstate Technologies), rabbit anti-Akt (Cell Signaling Technologies), rabbit anti-phospho-Akt (Cell Signaling), mouse anti-PINCH1 (BD Transduction), rabbit-ILK (Santa Cruz), rabbit anti-actopaxin/parvin (Sigma), mouse anti-p130CAS (Upstate), mouse anti-paxillin (BD Transduction), rabbit anti-c-src (Santa Cruz), mouse anti-vinculin (Sigma). The rabbit anti-Rsu-1 antibody was affinity purified and has been described (67).

Immunoprecipitations

Lysates from HC11-TRE CTGF cells and vector control HC11-TRE cells previously grown in the absence of doxycycline for 96 hours followed by maintenance for 48 hours in the absence of serum, and 15 or 30 minute prolactin stimulation (20μg/ml), were prepared in high salt RIPA buffer (283) containing AEBSF (20μg/ml), aprotinin (5μg/ml), leupeptin (5μg/ml), β-glycerol phosphate (100μM), and $NaVaO_4$ (1mM). For Stat5 immunoprecipitations, equal amounts of protein (500μg) were

incubated with 1μg/ml of primary antibody for 2 hours at 4°C and the immunoprecipitates were collected by binding to Protein A agarose beads (Invitrogen) for 3 hours at 4°C. Antibodies included anti-Stat5 (SantaCruz Biotechnology), and anti-phospho-Stat5 (Cell Signaling Technology). The process for western blot for this assay has been described previously (31) and as above.

MTT assay

The viability of HC11-TRE-CTGF and vector control HC11-TRE cells was determined by a proliferation assay using MTT dye (CellTiter96 Assay, Promega). Viable cells grown in the absence of doxycycline for 96 hours were replated at a density of 2.5 x 10^3 per well in quadruplicate wells of a 96-well plate in serum-free media with EGF (10ng/ml) and with or without CTGF/CCN2-containing conditioned media from HC11-TRE-CTGF cells and with or without an RGD-containing or control peptide (500μM) (BioMol) . The cells were incubated for 24, 48, 72, or 96 hours. Analysis of the MTT assay has been described previously (31). For analysis of metabolic activity, 15 ml of MTT dye solution was added to each well and the culture plate was incubated at 37ºC in a 5% CO_2 incubator for 4 hours. 100 μl of solubilization-stop solution was then added to each well and mixed evenly throughout. The plates were incubated in a humidified chamber overnight at room temperature and were then mixed by pipetting. The optical density was measured at 570 nm. The means and standard deviations of the absorbance values for the quadruplicate wells were calculated. Statistical significance was calculated by the student's *t*-test (2-tailed distribution and 2 sample unequal variance) and considered to be statistically significant with p values of < 0.05.

Cell cycle analysis by flow cytometry

HC11-TRE-CTGF and vector control HC11-TRE cells that had been grown in the absence of doxycycline for 96 hours were seeded at a density of 2 x 10^6 in 100mm plates in serum-free growth media with EGF (10ng/ml) and were harvested at 96 hours. Cells were scraped in PBS, pellets resuspended in 300μl PBS, 3ml methanol was added dropwise for fixation, and cells were stored at -20°C for 24-96 hours. The cells were pelleted for 5 minutes at 1500 rpm, methanol was removed, and the cells were resuspended in 1ml cold PBS. Propidium iodide (50μg/ml) was added to the cells 30 minutes to 24 hours prior to flow cytometric analysis. Cell cycle was determined on a BD Bioscience LSRII cytometer (BD Biosciences), followed by analysis with ModFit LT software (Verity Software House, Inc.).

TUNEL Assay

The event of apoptosis was detected in vector control HC11-TRE cells and HC11-TRE-CTGF cells by TUNEL technology (*In Situ* Cell Death Detection Kit, Fluorescein, Roche). Cells previously grown in the absence of doxycycline for 96 hours were grown on coverslips in serum-free media in the presence of EGF (10ng/ml) for 96 hours. The cells were briefly air dried on the coverslips prior to fixation in 4% paraformaldehyde for 1 hour at room temperature. The cells were rinsed briefly in 1X PBS and permeabilized in 0.1% Triton X-100 for 2 minutes on ice, followed by another two rinses in 1X PBS. 50μl of TUNEL reagent (prepared according to manufacturer's instructions) was added to each coverslip for 1 hour at 37°C in the dark. The cells were rinsed three times in 1X

PBS prior to being mounted on slides (Vectashield mounting media, Vector Labs). Cells were viewed on the Olympus BX61 and analyzed by IVision software (IVision).

Immunofluorescence

To detect focal adhesions, HC11-TRE and HC11-TRE-CTGF cells were grown in the absence of doxycycline for 96 hours, at which time they were seeded in serum-free media with EGF (10ng/ml) for 96 hours. The cells were washed with PBS twice and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed with PBS and permeabilized with 0.5% Triton X-100 for 10 minutes at room temperature. Cells were washed with PBS and blocked for 1 hour in 4% BSA followed by an overnight incubation at 4°C with an anti-vinculin antibody (Sigma).

To detect the localization of Stat5, HC11-TRE and HC11-TRE-CTGF cells were grown in growth media with EGF (10ng/ml), and serum-starved for 48 hours in the absence of EGF. The cells were then stimulated with 20μg/ml prolactin and fixed in 1% paraformaldehyde at the times indicated. The cells were washed in PBS, permeabilized in 1:1 methanol:DMSO, blocked in 5% FBS, and incubated with mouse anti-Stat5 (BD Transduction) overnight. For both focal adhesion and Stat5 localization experiments, cells were washed with PBS and incubated for 30 minutes in the dark at room temperature with an AlexaFluor 488-conjugated secondary antibody. The cells were washed again in PBS and mounted to slides with ProLong Gold antifade reagent containing DAPI (Molecular Probes, Invitrogen). Immunofluorescence was visualized using an Olympus IX71 microscope, a QImaging Retiga 2000RV camera, and QCapture Pro 6.0 software (Olympus America Inc.).

Detection of β1 and α6 Integrins by Flow Cytometry

Surface level expression of β1 and α6 integrins was determined by flow cytometry. HC11-TRE and HC11-TRE-CTGF cells previously grown in the absence of doxycycline for 96 hours were seeded in 100mm tissue culture dishes for 96 hours in serum-free growth media with EGF (10ng/ml). The cells were removed from the plate with Cell Stripper (CellGrow), pelleted, washed, and counted. The cells were aliquoted into 1ml samples of 500,000 cells each and washed in 5ml of FACS was buffer (1% FBS, 0.05% sodium azide). Cells were then incubated in 150µl of buffer with either a primary antibody or the isotype control for 1 hour at 4ºC. The cells were pelleted, resuspended in 50µl of buffer containing PE- or FITC- conjugated secondary antibodies for 45 minutes at 4ºC. The cells were again pelleted and fixed in 300µl of Cytofix (BD Biosciences) for 15 minutes on ice. Again the cells were pelleted, washed in 1X PBS, and resuspended in 500µl of FACS buffer. Flow cytometry was performed on a BD Biosciences LSRII cytometer and analysis was performed using WinList software (Verity Software House Inc.).

Chromatin Immunoprecipitations

To detect the level of binding of relevant proteins to the β-casein promoter, chromatin immunopreciptation assays (ChIP assays) were performed on HC11-TRE and HC11-TRE-CTGF cells. The cells that had been previously grown for 96 hours in the absence of doxycycline were seeded in 150mm plates and let grow to confluence. Once confluent, the cells were grown in media without EGF for 24 hours. The cells were then stimulated with DIP for 16 hours prior to cross-linking with 1% formaldehyde for 10

minutes at room temperature. To quench the formaldehyde, the cells were treated with 1.12ml of 2.5M glycine for 5 minutes at room temperature. The method for the ChIP assay was performed as described (27) with modifications. Briefly, the cells were kept on ice, washed 3X with 1X PBS containing protease inhibitors PMSF (1μg/ml), aprotinin (5μg/ml), leupeptin (5μg/ml), and harvested. Cells were pelleted for 5 minutes at 2000rpm at 4ºC and resuspended in cell lysis buffer (25mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP40, 1mM DTT) and left on ice for 10 minutes prior to douncing with a type B homogenizer. Lysates were centrifuged for 4 minutes at 2000 rpm at 4ºC and the pellets were resuspended in 500μl of sonication buffer (50mM HEPES, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% DOC, 0.1% SDS). Nuclei were sonicated to shear DNA to average lengths of 500bp. Sonication was performed using the Misonix S-4000 (Misonix Inc., Farmingdale, NY) with microtip in 10 second pulses with 15 seconds incubation on ice between pulses for 10 pulses. Sonicated samples were centrifuged for 15 minutes at 14,000rpm at 4ºC and the supernatants were diluted to 1.2ml in sonication buffer. 3% of the diluted sample was removed and used to determine shearing efficiency as well as for use as control input DNA, which would be processed with the eluted DNA from the immunoprecipated samples. Remaining samples were aliquoted to 600μl, pre-cleared with protein A bead/salmon sperm slurry and incubated overnight on ice at 4°C with 5μg of anti-rabbit IgG (Chemicon, PP64) or anti-Stat5 N-term (Santa Cruz, sc-836). Immunocomplexes were collected with protein A agarose beads (Invitrogen) for 2 hours with rotation at 4ºC. Beads were washed with sonication buffer, followed by washes with high salt buffer (50mM HEPES, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% DOC, 0.1% SDS) and LiCl buffer (20mM Tris, 1mM EDTA, 250mM LiCl, 0.5%

NP40, 0.5% DOC), and Tris-EDTA buffer. DNA was eluted from the beads with elution buffer (50mM Tris, 1mM EDTA, 1% SDS) at 65°C, and cross-links were reversed overnight at 65ºC, followed by 45 minute incubation at 42ºC with 2µl proteinase K (10mg/ml) and 20µl 0.1M EDTA. DNA was extracted by phenol/chloroform and ethanol precipitated in the presence of 20µg glycogen overnight. 5µl of DNA was used for each PCR sample, including 5µl of input DNA that had been diluted 4-fold. Primers for the proximal promoter of β-casein have been described (27) and include: Fwd 5' CCAGCTTCTGAATTGCTGCC 3', Rev 5' GGTCTATCAGACTCTGTGAC 3'. PCR conditions included 35 cycles of 1 minute at 95ºC, 30 seconds at 55ºC, 30 seconds at 72ºC. Amplified DNA was analyzed by electrophoresis on an ethidium bromide stained 1.5% agarose gel. Bands were quantitated using the Fujifilm LAS-4000 (Fujifilm Medical Systems USA, Inc.). Results are expressed as a ratio of immunoprecipitated DNA to the input DNA.

Adhesion Assay

To determine the ability of cells to adhere to CTGF/CCN2 in an integrin-dependent manner, adhesion assays were performed as described (154) with a few modifications. Maleic anhydride Reacti-Bind microtiter plates (Thermo Scientific) were coated with 50µl of 2µg/ml human recombinant CTGF/CCN2 (Cell Sciences) overnight at 4°C. The plates were then blocked with 1% BSA for 2 hours at 37°C. HC11-TRE cells were trypsinized, counted, and resuspended at a concentration of $5x10^5$ cells/ml in serum-free growth media containing EGF (10ng/ml). After blocking, the BSA was removed from the plates and 50µl of cell suspension was plated and the cells were

allowed to attach for 4 hours at 37°C. Where indicated, the cell suspension was mixed with EDTA (2.5mM), EDTA + Mg^{2+} (5mM), rat anti-α6 integrin (25μg/ml, BD Biosciences) or rat isotype control (Chemicon), hamster anti-β1 integrin (25μg/ml, BD Biosciences), hamster isotype control (25μg/ml, BD Biosciences), hamster anti-β3 or hamster anti-αv (25μg/ml, Santa Cruz Biotechnology). After 4 hours, the unattached cells were carefully removed and the wells were washed with 1X PBS. The cells were then fixed with 3.7% formaldehyde for 30 minutes at room temperature, followed by overnight staining with 0.1% crystal violet. The plates were washed lightly under running tap water, let dry at room temperature, and the absorbance was read at 570nm. The means and standard errors of the absorbance values for quadruplicate wells were calculated. Statistical significance was calculated by the Student's *t*-test (2-tailed distribution and 2 sample unequal variance).

Chapter 3: Results

Part 1: The Requirement of Connective Tissue Growth Factor (CTGF/CCN2) for Lactogenic Differentiation

The first part of this chapter focuses on preliminary data leading up to the formation of the hypothesis for this study. We initially found the upregulation of CTGF/CCN2 in HC11 cells by microarray analysis. We confirmed this finding by investigation of CTGF/CCN2 mRNA and protein expression in the developing mouse mammary glands. Since CTGF/CCN2 was upregulated in response to stimulation with DIP, we investigated its role in the transcription of β-casein, both in HC11 cells and in primary mammary epithelial cell cultures. The findings from Part 1 contributed to the hypothesis that CTGF/CCN2 is a regulator of lactogenic differentiation.

CTGF/CCN2 is differentially expressed in the developing mouse mammary gland

Our lab has worked extensively on the mechanisms involved in lactogenic differentiation (31, 82, 270, 271). Microarray analysis led to the discovery that Connective Tissue Growth Factor (CTGF/CCN2) is one of the most highly upregulated proteins in HC11 mouse mammary epithelial cells that had been stimulated to undergo lactogenic differentiation (271). Since CTGF/CCN2 is not commonly studied in the mammary gland, confirmation of the microarray results was required. It was determined that CTGF/CCN2 mRNA is expressed in the mammary glands of virgin, pregnant, lactating, and involuting mice by northern blot analysis of RNA from mouse mammary glands (271). The levels of mRNA increased throughout pregnancy and dropped off

after day 1 of lactation, and reappeared during the late stages of involution, confirming our microarray analysis. To confirm the RNA analysis, immunohistochemical analysis was performed to determine any possible differential localization of CTGF/CCN2 in the glands of virgin, pregnant, and involuting mice (**Figure 1**). Staining of sectioned mouse mammary glands for CTGF/CCN2 detected slight positive staining in the epithelial cells lining the duct and more intense periductal stromal expression in the virgin glands (**Figure 1a, b**). In the pregnant gland (**Figure 1c,d**) there is considerably enhanced intraductal epithelial expression of CTGF/CCN2, which can be particularly seen in the higher magnification image. In the pregnant gland the basement membrane and stromal components are typically difficult to detect between the acinar structures (137), so this suggests that the staining seen in this image is primarily epithelial, though periductal stromal expression of CTGF/CCN2 is also evident similarly to the virgin gland. During mammary gland involution, which initiates at the conclusion of weaning, the epithelial secretory structures present during pregnancy and lactation begin to undergo apoptosis and the structures themselves appear to shrivel and condense (43). Thus, intense ductal epithelial and light stromal reactivity was observed in the tissue from the involuting glands (**Figure 1e, f**). The staining pattern suggests that CTGF/CCN2 is expressed in the mammary gland, supporting the data obtained from the microarray and RNA analysis (271).

CTGF/CCN2 is required for lactogenic differentiation

Because we found that CTGF/CCN2 is highly upregulated specifically in response to dexamethasone treatment, we wanted to determine its effect on the

transcription of β-casein, a predominant milk protein. We used multiple methods to determine the effect. First, we were able to transiently transfect HC11 cells with vectors encoding CTGF/CCN2. After stimulation for 24-72 hours with the lactogenic hormone mix of dexamethasone, insulin and prolactin (DIP), northern blot analysis showed that β-casein transcription was significantly enhanced in cells transfected with CTGF/CCN2 compared with the control (271). To confirm this, the same vectors were transfected into HC11-luci cells. This cell line contains a luciferase reporter fused to the β-casein promoter, such that the amount of luciferase produced is a direct correlation to the activation of the β-casein promoter (31). After stimulation with DIP for 48 hours, CTGF/CCN2-transfected cells showed higher levels of β-casein promoter activity compared with the vector control (271).

HC11 cells were then transfected with an siRNA construct specific to CTGF/CCN2 in order to knock down its expression (271). After treatment of the cells with DIP, northern blot analysis showed that a decrease in CTGF/CCN2 mRNA expression corresponded to a decrease in β-casein mRNA expression, suggesting that CTGF/CCN2 is required for the initiation of β-casein transcription (271). Together these results confirm that CTGF/CCN2 not only enhances, but is required for the lactogenic differentiation of HC11 mouse mammary epithelial cells.

Figure 1

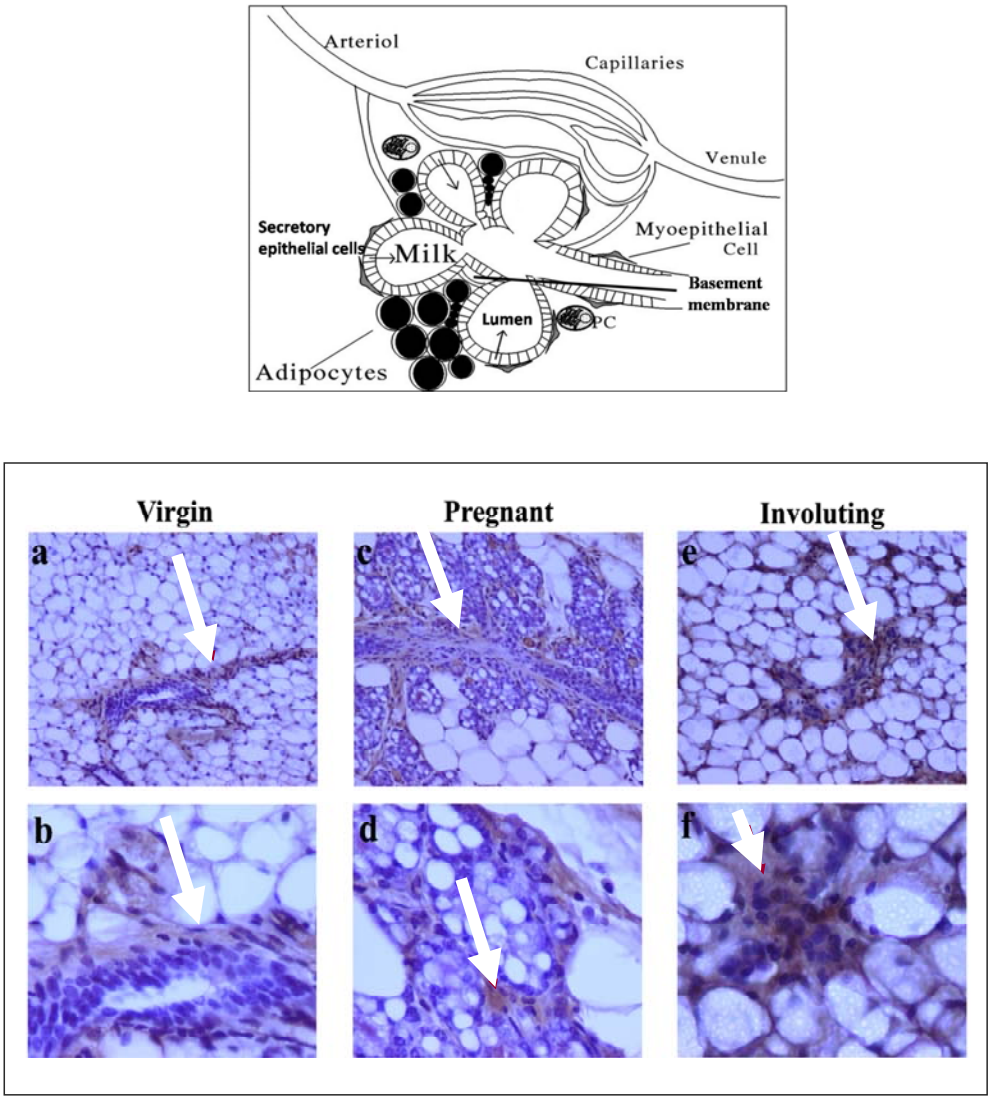


Figure 1. ***CTGF/CCN2 is expressed in the mouse mammary gland***. ***Top***: Diagram of the cells of the mouse mammary gland. *Adapted from http://mammary.nih.gov/reviews/lactation/Neville001/index.html*. ***Bottom***: Sections of mammary glands from FVB mice were paraffin embedded and analyzed by immunohistochemistry using a goat anti-CTGF antibody and counterstained in hematoxylin. a,b: One-year-old virgin. c,d: Pregnant day 18. e,f: Involuting day 10. Magnification: 10X (a,c,e) and 40X (b,d,f). Positive CTGF/CCN2 expression is displayed as a brown stain. White arrows point to examples of positive staining in each tissue (271).

Ectopic expression of CTGF/CCN2 in primary mammary epithelial cells enhances β-casein transcription

Our initial findings of the effects of CTGF/CCN2 on lactogenic differentiation were done using the HC11 cell line. In order to ensure that this effect is not specific to that cell line, our findings were extended to primary mouse mammary epithelial cells. A replication defective adenovirus expressing CTGF/CCN2 was used to transiently express CTGF/CCN2 in primary mammary epithelial cells isolated from the mammary glands of pregnant FvB/N mice. The detection of β-casein mRNA by semi-quantitative RT-PCR and real-time PCR was used as a measure of differentiation. The infected primary cells were induced to differentiate with DIP in the absence of EGF. As expected, the results showed an increase in the level of β-casein mRNA expression in the primary cells infected with the CTGF/CCN2 adenovirus compared to the vector control infected cells at 24 hours post induction (**Figure 2**). These results demonstrated that the effect of CTGF can be seen during lactogenic differentiation in the mouse mammary gland as well as in the HC11 cells.

Figure 2

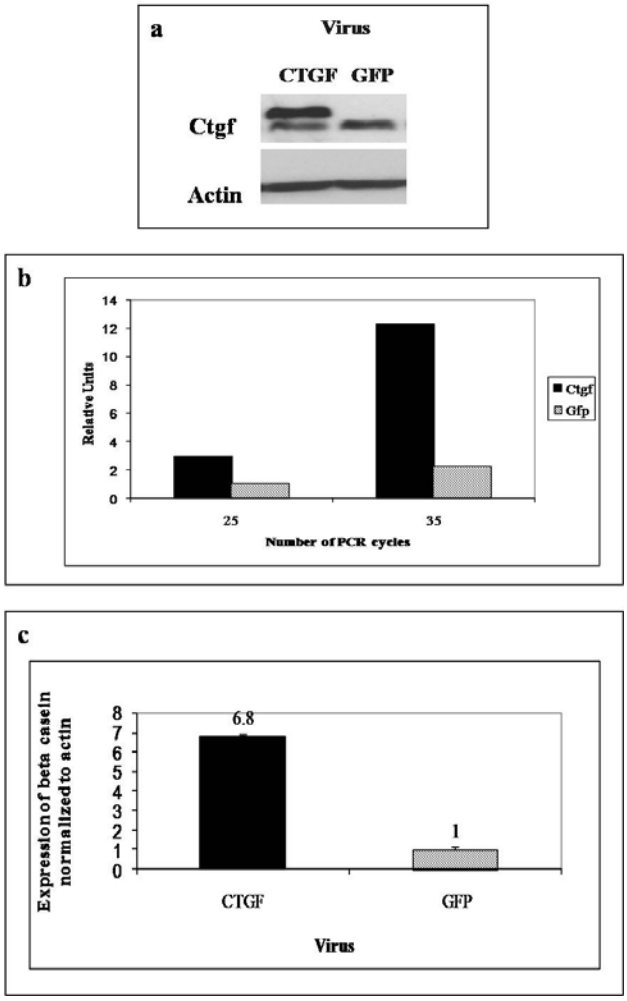


Figure 2. ***CTGF/CCN2 enhances β-casein transcription in primary mouse mammary epithelial cells.*** Primary cultures of mouse mammary epithelial cells established from pregnant mice were infected with CTGF-encoded or GFP vector control adenovirus in media containing 10% FBS, but no EGF. Five hours post infection; the cells were stimulated with DIP for 24hrs. (**a**) Protein was isolated and the levels of vector-encoded (upper band) and endogenous CTGF/CCN2 were determined. RNA was isolated and the levels of β-casein and actin mRNA were determined by quantitative RT-PCR (**b**) and real-time PCR (**c**). Expression levels of β-casein in (b) and (c) were normalized to actin. Results in (c) are expressed as the mean $\pm$ S.D. of triplicate readings.

Part 2: Role of CTGF/CCN2 in Lactogenic Differentiation

In part 1 of this chapter, it was established that CTGF/CCN2 is required for lactogenic differentiation, and also that this effect holds true in primary mammary epithelial cell cultures. In order to now investigate the mechanism of CTGF/CCN2 in HC11 cells, a method was developed such that the expression of CTGF/CCN2 could be stably regulated without any additional factors. To do this, a stable HC11-based cell line containing CTGF/CCN2 under the control of a Tet-responsive promoter was created, such that CTGF/CCN2 expression would be induced upon the removal of doxycycline from the culture media. Using this cell line, the effect of CTGF/CCN2 on multiple parameters of lactogenic differentiation was determined. We then moved further into the mechanism by investigating the effect of CTGF/CCN2 on the activation of the β-casein promoter both as a result of transcription factor activity and on the ability of the cells to generate enhanced activation signals in response to additional matrix proteins. The results from Part 2 of this chapter confirm that CTGF/CCN2 contributes to lactogenic differentiation as a result of interaction with the extracellular matrix and the enhanced activity of the Stat5 transcription factor.

Ectopic CTGF/CCN2 expression in HC11 cells.

The goal of the current study is to elucidate the mechanism by which CTGF/CCN2 enhances the lactogenic differentiation of mammary epithelial cells. A stable HC11-based mouse mammary epithelial cell line with inducible expression of CTGF/CCN2 was created for use in this study. This stable cell line contained a pREV-TRE (Clontech) retroviral vector expressing a Tet-regulated HA-tagged open reading

frame of CTGF. Thus, CTGF/CCN2 was under the control of a Tet-responsive promoter in a Tet-off system (HC11-TRE-CTGF) such that the expression of CTGF/CCN2 increased following the removal of doxycycline from the culture media. Several independent clones that exhibited inducibility of CTGF/CCN2 expression following the removal of doxycycline were isolated. The regulation of the expression of CTGF/CCN2 protein in three representative clones is shown in **Figure 3**. The selected clones showed little or no expression of CTGF/CCN2 in the presence of only doxycycline, enhanced expression of only the dexmethasone-mediated levels of CTGF/CCN2 upon treatment with DIP in the presence of doxycycline, as well as the expression of the vector-encoded CTGF/CCN2 upon the removal of doxycycline. Vector-encoded CTGF/CCN2 appears as a slightly higher molecular weight band due to the HA tag. Because its CTGF/CCN2 expression was most regulated by doxycycline, Clone 2 was selected and used for all further experimentation. To evaluate the effect of CTGF/CCN2 on lactogenic differentiation, both HC11-TRE vector control and HC11-TRE-CTGF cells were grown in the absence of doxycycline for 96 hours prior to seeding for experimental purposes. The effect of CTGF/CCN2 on known markers of lactogenic differentiation, such as β-casein transcription, Stat5 phosphorylation, and mammosphere formation, were established using these cell lines. The results confirm and extend our previous findings of the contribution of CTGF/CCN2 to this process (271), which includes its necessary involvement for β-casein transcription in HC11 cells.

Figure 3

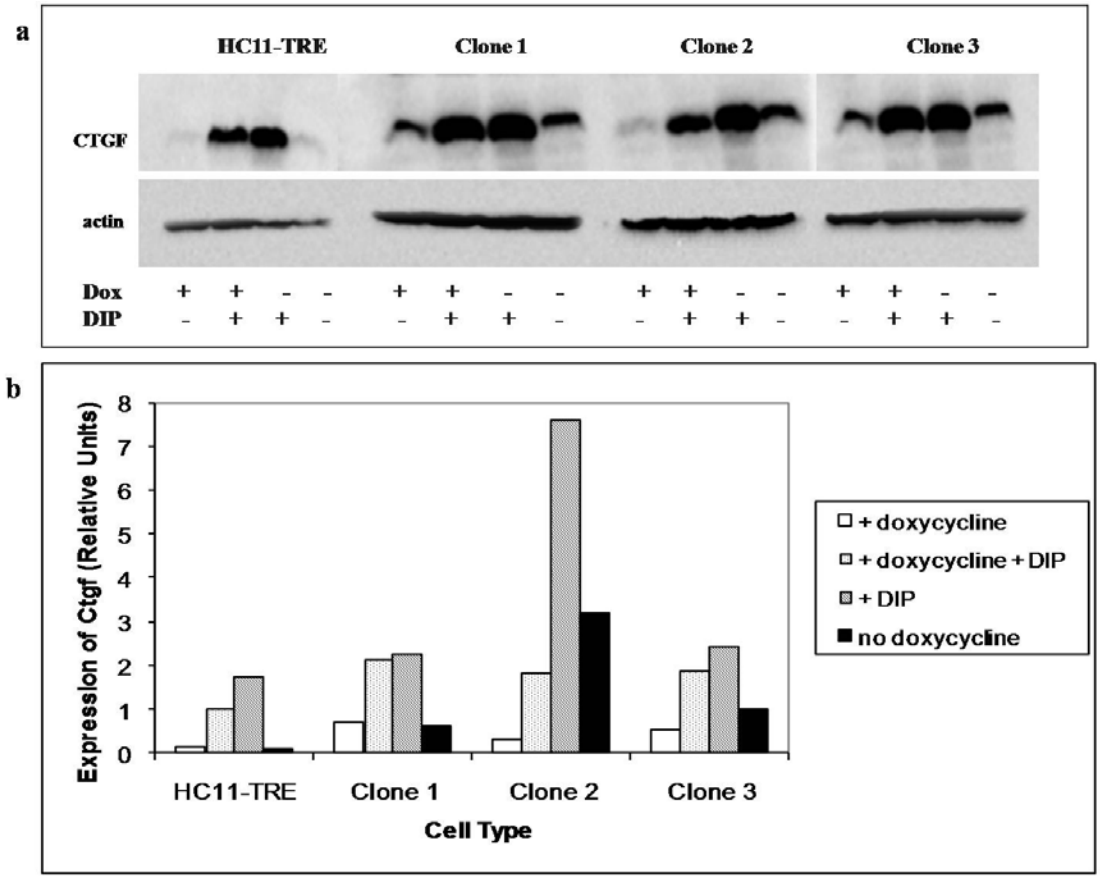


Figure 3. ***CTGF/CCN2 expression upon the removal of doxycycline in HC11-TRE cells.*** The HC11-TRE vector control cell line was used to express HA-CTGF in response to the removal of doxycycline (Dox) as described previously (271). Twenty-four colonies from HC11 Tet-off infected cells were isolated and expanded into cell lines. The clonal cell lines were tested for expression of vector encoded HA-CTGF protein following the removal of doxycycline from the culture media. (**a**) The protein expression levels of CTGF/CCN2 or actin with or without doxycycline as well as with or without DIP (dexamethasone, insulin, prolactin) treatment are shown from three representative clones and compared to the vector control HC11-TRE line. (**b**) Quantification of the images in (a) by densitometry. Results are reported as units normalized to actin and are representative of selected clones, each determined by n=1.

CTGF/CCN2 enhances mammosphere formation

To characterize the HC11-TRE-CTGF cell line and also to begin to understand the mechanism of CTGF/CCN2 in mammary epithelial cells, its effect on multiple parameters of lactogenic differentiation was determined. As a morphological marker, the effect of CTGF/CCN2 expression on mammosphere formation was determined. Mammospheres are characterized as lipid-containing domed structures that can be formed when a confluent and competent layer of mammary epithelial cells is continuously stimulated with DIP (31). Following the growth of HC11-TRE-CTGF and HC11-TRE cells to confluence, EGF was removed from the media due to its ability to block the response to lactogenic hormones (82). Differentiation was induced by the addition of DIP. Differentiation of the cells was observed at 120 hours post-DIP induction when the mammospheres were visualized and photographed using phase-contrast microscopy and manually enumerated (**Figure 4**). The results indicated that enhanced mammosphere formation, both in size and number, was displayed by HC11-TRE-CTGF cells.

CTGF/CCN2 enhances β-casein transcription in HC11 cells

The effect of CTGF/CCN2 on the early transcription of β-casein was then determined. Previously, our results followed the effect of CTGF/CCN2 on β-casein transcription at 24 or 48 hours post-DIP induction. Earlier time points were assayed in this study because it has since been determined that CTGF/CCN2 is rapidly induced by dexamethasone, a component of the lactogenic hormone stimulation mix. Confluent cultures of HC11-TRE-CTGF and HC11-TRE cells were stimulated with the lactogenic hormone mix for 0, 8, or 16 hours and assayed for differentiation by following the levels

of β-casein mRNA expression. RNA was harvested from the cells and used to determine the level of β-casein mRNA expression by RT-PCR and Southern blot analysis (**Figure 5**). The results indicated that CTGF/CCN2 enhanced β-casein expression at 8 hours compared to levels seen in the HC11-TRE cells. In addition, elevated CTGF/CCN2 expression resulted in detectable β-casein in the absence of hormone (T=0). These results demonstrated that CTGF/CCN2 expression enhances the early transcription of β-casein in the HC11 cell line.

Figure 4

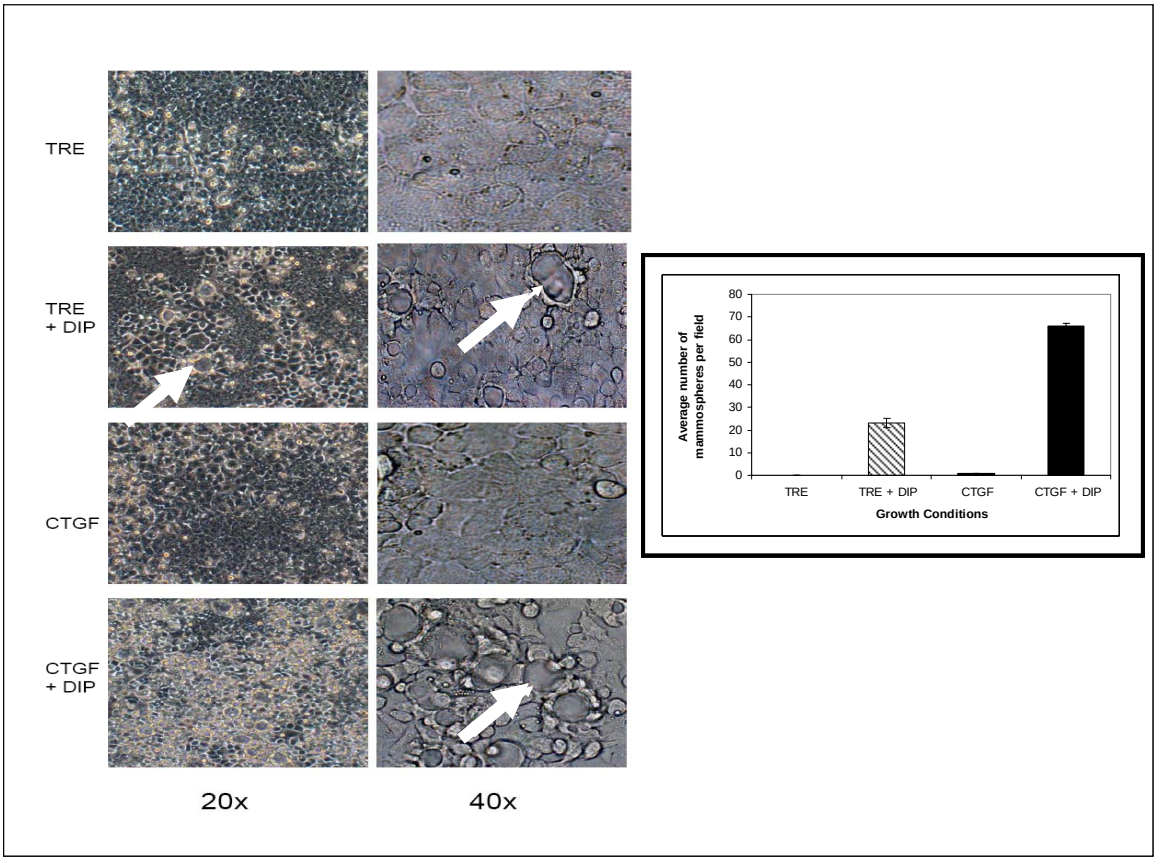


Figure 4. ***CTGF/CCN2 contributes to the formation of mammospheres.*** HC11-TRE and HC11-TRE-CTGF cells were grown to confluence and exposed to DIP as described in Materials and Methods. The cells were photographed at 120 hrs post-DIP addition. The images shown representative of five fields. Mammospheres are shown as bubble-like structures, indicated by white arrows. The results of mammosphere enumeration at 20X are shown to the right. Mean is the result of the average number of mammospheres in 5 microscopic fields.

Figure 5

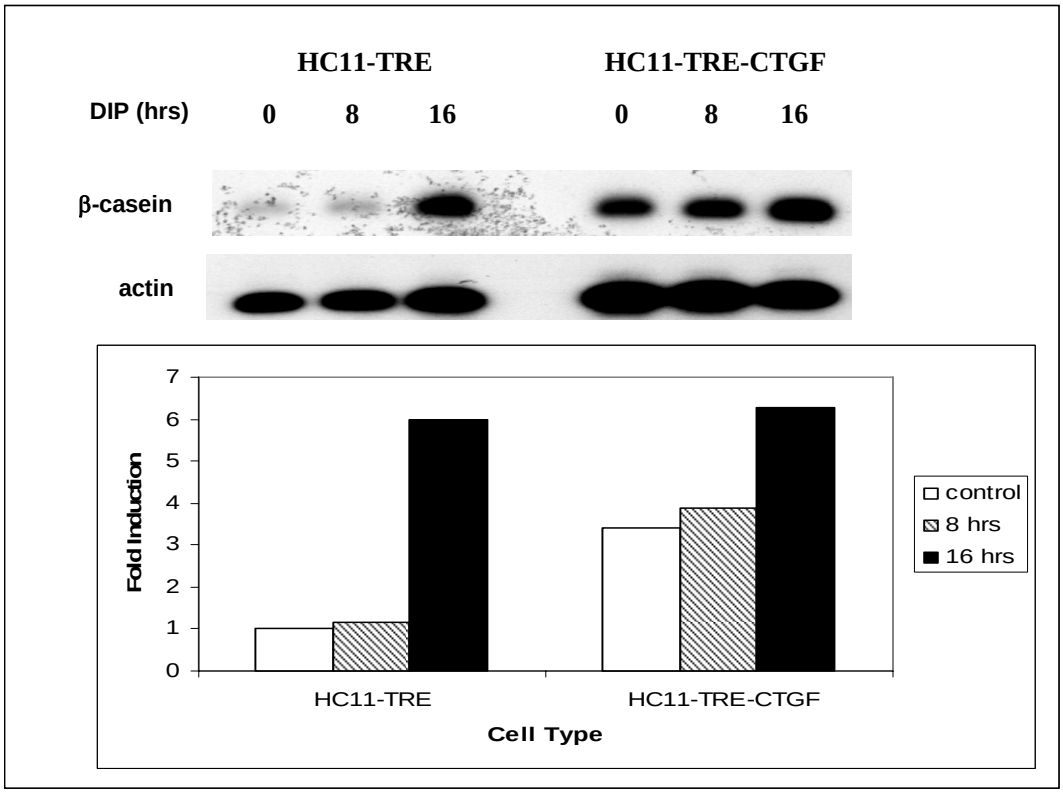


Figure 5. ***CTGF/CCN2 enhances β-casein transcription in HC11 cells.*** HC11-TRE and HC11-TRE-CTGF cells were stimulated with DIP for 0, 8, or 16 hours. RNA was extracted and examined by Southern blotting for expression of β-casein and actin. For quantitation, expression levels of β-casein were normalized to actin and expressed as a fold induction of β-casein mRNA compared to the HC11-TRE control. The trend in data is representative of two experiments.

Elevated expression of full length CTGF/CCN2 is required for the enhancement of β-casein

Before getting deep into the mechanism of CTGF/CCN2, one question that had to be addressed was whether the effect of CTGF/CCN2 seen was the result of the full-length protein or one of its cleavage products. CTGF/CCN2 is composed of unique domains. Beginning at the N-terminus with a signal sequence, domain 1 is an IGF binding protein (IGFBP) homology domain, domain 2 is a von Willebrand type C repeat (vWC), domain 3 resembles thrombospondin 1 (TSP-1), and domain 4, the carboxyl-terminal (CT) domain, contains a cysteine knot motif (18, 149). As previously mentioned, CTGF/CCN2 can undergo cleavage at the hinge region between the N-terminal region and the C-terminal region, resulting in variant proteins with potentially diverse functions. CTGF/CCN2 can also be cleaved between each individual domain, possibly resulting in differentially functional proteins. In order to determine whether the CTGF/CCN2-mediated enhancement of β-casein is mediated by the full length protein or a cleavage product, HC11 cells were infected with GFP-encoded adenoviral vectors expressing domain variants of CTGF/CCN2, as shown in **Figure 6a**. The cells were also infected with a control GFP virus as well as the full-length CTGF/CCN2 virus used in previous publications from our lab (271). The cells were then induced with DIP for 24 hours and the levels of CTGF/CCN2 and β-casein mRNA were determined by northern blot analysis. The results in **Figure 6b** show the expected levels of endogenous CTGF/CCN2, which are elevated when cells are treated with DIP. The results also show the levels of vector-encoded CTGF/CCN2, which is at a slightly smaller size due to only

the open reading frame being cloned into the viral vector. It is also possible to see the sizes of the CTGF/CCN2 variant RNA, which are depicted by the numbers given to the variant vectors in **Figure 6a**. The level of β-casein mRNA is increased as expected in cells that were treated with DIP and significantly enhanced in the cells infected with the full length CTGF/CCN2-encoded adenovirus. The expression of β-casein mRNA is not enhanced to the same level in the cells infected with the variant proteins as is seen in the cells infected with the full length virus. This suggests that the full length CTGF/CCN2 is required for the level of β-casein transcription previously seen in response to CTGF/CCN2.

Figure 6

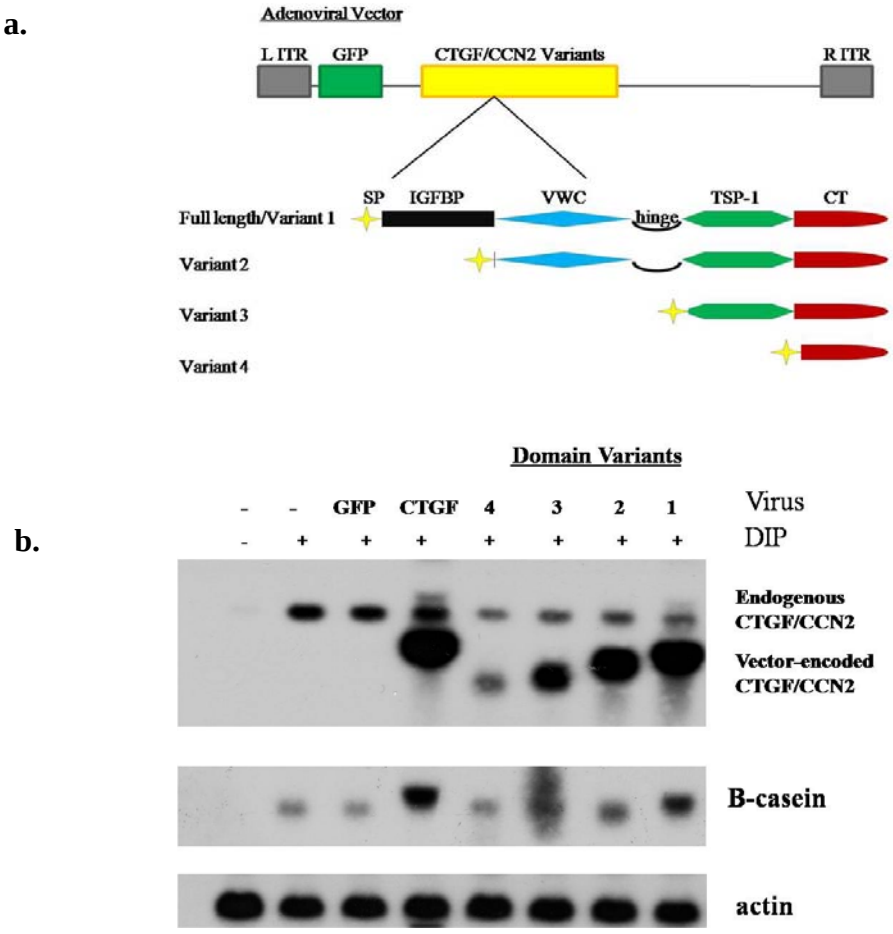


Figure 6. ***Full length CTGF/CCN2 is required for enhanced β-casein transcription.*** (***a***) Diagram of adenoviral vector and CTGF/CCN2 variant structures. LITR: left inverted terminal repeat. RITR: right inverted terminal repeat. (***b***) HC11 cells were infected with GFP-encoded CTGF/CCN2 adenoviral vector variants and subsequently treated with DIP for 24 hours. Total RNA was isolated and levels of CTGF/CCN2 and β-casein mRNA were determined by northern blot analysis. GFP: GFP control virus, CTGF: full length CTGF-encoded adenovirus.

CTGF/CCN2 contributes to the activation of Stat5 in HC11 cells

To identify the mechanism of CTGF/CCN2 at the β-casein promoter, its effects on Stat5 activity were determined. The transcription of β-casein requires the activation of the prolactin receptor and Janus kinase2 (Jak2)/signal transducers and activators of transcription 5 (Stat5) pathway (92). The action of prolactin binding to the prolactin receptor triggers the recruitment and dimerization of Jak2, which then phosphorylates both the prolactin receptor and the Stat5 transcription factors (5). Stat5 then forms homo- or heterodimers and translocates to the nucleus where it can bind to the promoter of its target gene (4). The activation of this pathway is known to be dependent on interactions with components of the extracellular matrix (246). Thus, as a measure of the effect of CTGF/CCN2 on the activity of prolactin signaling, tyrosine phosphorylation of Stat5 in response to prolactin was determined. HC11-TRE and HC11-TRE-CTGF cells were maintained in the absence of serum for 48 hours and then stimulated with prolactin in the absence of EGF for 0, 15, or 30 minutes. The status of Stat5 phosphorylation was determined by the immunoprecipitation of total Stat5 and western blot using anti-Stat5 Tyr694 phosphorylation-specific antibodies (**Figure 7**). The results showed that the phosphorylation of Stat5 was elevated and sustained in the HC11-TRE-CTGF cells compared to the HC11-TRE cells. These results suggest that CTGF/CCN2 is contributing to either the activation of the prolactin receptor-mediated signaling pathway leading to Stat5 phosphorylation or to the regulation of Stat5 phosphorylation and desphosphorylation. To confirm these findings, the effect of CTGF/CCN2 on the localization of Stat5 was determined by immunofluorescence (**Figure 8**). **Figure 8a** shows the quantitation of the percentage of cells displaying nuclear localization of Stat5,

while **Figure 8b** depicts immunofluorescent staining of Stat5 after 45 minutes of treatment with prolactin. It was found that Stat5 localization to the nucleus is enhanced in HC11-TRE-CTGF cells compared to the HC11-TRE control cells and is sustained through 45 minutes of prolactin stimulation.

Together with enhanced mammosphere formation, these results demonstrated that an increase in lactogenic differentiation is observed upon ectopic expression of CTGF/CCN2 in HC11 cells. Moreover, this CTGF/CCN2-mediated enhancement of lactogenic differentiation correlates with prolonged Stat5 phosphorylation and nuclear localization.

Figure 7

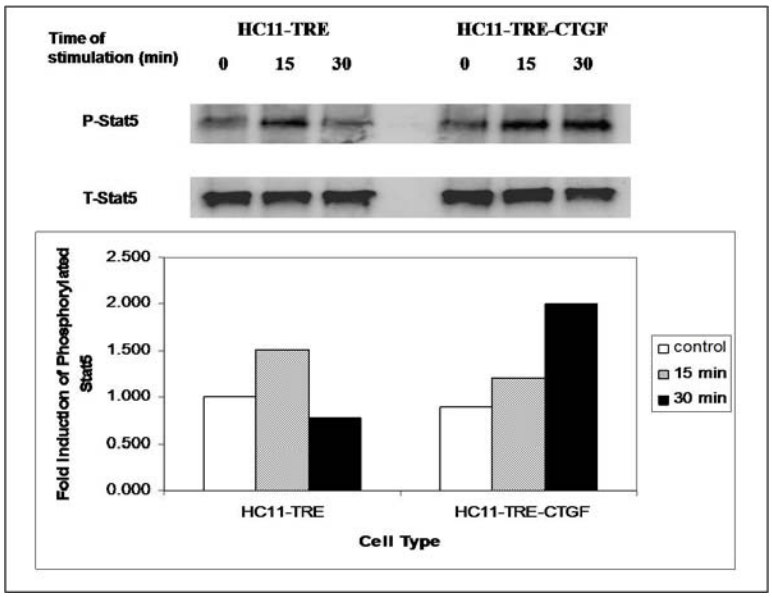


Figure 7. ***CTGF/CCN2 contributes to the phosphorylation of Stat5.*** HC11-TRE and HC11-TRE-CTGF cells were serum- and EGF-starved for 48 hrs prior to stimulation with prolactin (20μg/mL) for 0, 15, or 30 minutes. Total Stat5 was immunoprecipitated and levels of phosphorylated Stat5 and total Stat5 were determined by western blot. The amount of phospho-Stat5 was normalized to levels of total Stat5 and reported as fold induction of phosphorylated Stat5. The data trend shown is representative of three experiments.

Figure 8

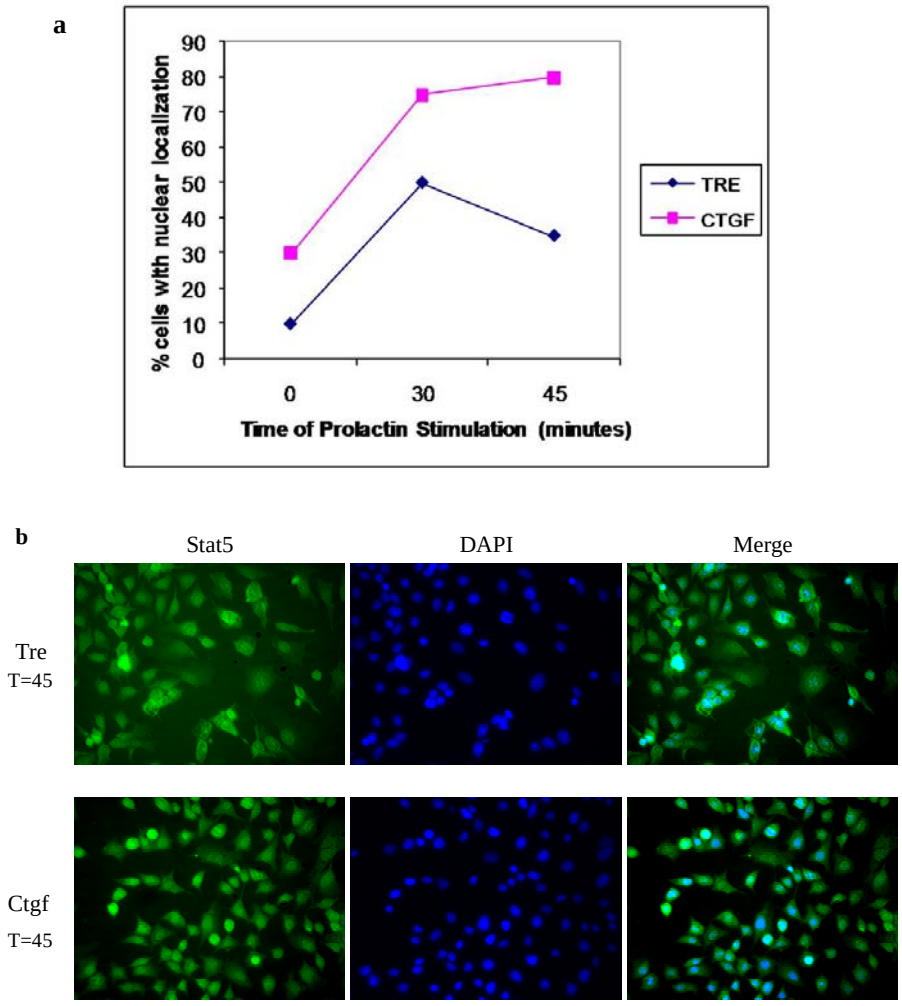


Figure 8. ***CTGF/CCN2 expression enhances the nuclear localization of Stat5.*** HC11-TRE and HC11-TRE-CTGF cells were maintained in the absence of serum and EGF for 48 hrs prior to stimulation with prolactin (20μg/mL) for 30 and 45 minutes. The cells were washed with PBS and fixed with 4% paraformaldehyde prior to being incubated with an anti-Stat5 antibody. The anti-Stat5 antibody was detected with an AlexaFluor 488 secondary antibody such that positive binding to Stat5 appears green in the above images. The cells were also stained with DAPI for nuclear detection. Secondary controls were also run (data not shown). (**a**) Percentage of cells displaying nuclear localization of Stat5 per 100 counted cells, (**b**) Immunofluorescence image from 45 minutes of stimulation with prolactin. Left, Stat5; middle, DAPI; right, merged. The images are representative of two independent experiments.

CTGF/CCN2 enhances the binding of Stat5 to the β-casein promoter

This research has demonstrated that CTGF/CCN2 enhances the activation of β-casein transcription as well as the activation of Stat5. To determine the effect of CTGF/CCN2 on the binding of Stat5 to the β-casein promoter, a chromatin immunoprecipitation assay was performed on HC11-TRE and HC11-TRE-CTGF cells. The cells were grown to confluence and the EGF was removed from the media for 24 hours prior to stimulation with DIP for 16 hours. Cells were fixed in formaldehyde and the nuclei isolated from the lysates were sonicated, followed by incubation with antibodies against the N-terminus of Stat5. The cross-links were reversed and the β-casein proximal promoter was amplified from purified DNA in order to determine the level of DNA binding of Stat5. Results, shown in **Figure 9,** revealed that elevated CTGF/CCN2 expression led to an increase in the binding of Stat5 on the β-casein proximal promoter. This finding suggests that CTGF/CCN2, by stabilizing the level of β1 integrin activation required to initiate Stat5 translocation, contributes to the DNA binding required for full activation of the β-casein promoter.

Figure 9

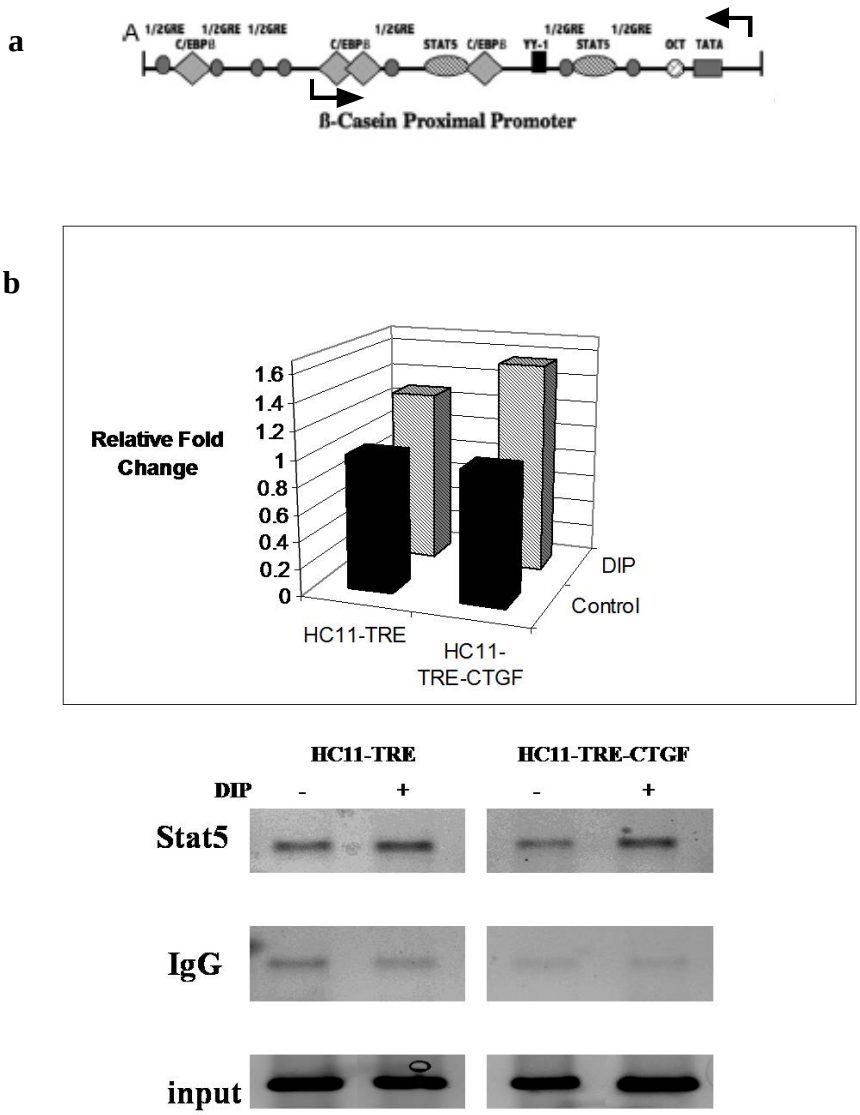


Figure 9. ***CTGF/CCN2 contributes to the DNA binding ability of Stat5***. (**a**) Diagram of the β-casein proximal promoter depicting the binding sites for Stat5 and other DNA binding proteins as well as the promoter sites used in the chromatin immunoprecipitation in (b), shown by black arrows. *Adapted from Kabotyanski et al.* (125). (**b**) HC11-TRE and HC11-TRE-CTGF cells grown under serum-free conditions for 24 hours prior to being induced with DIP for 16 hours. ***Top***: Graphical analysis of DNA immunoprecipitated with an anti-Stat5 antibody, normalized to the amount of input DNA. ***Bottom***: PCR analysis of DNA immunoprecipitated with either an anti-Stat5 antibody or control IgG. Results are representative of three experiments.

CTGF/CCN2 contributes to the epithelial-matrix interaction during lactogenic differentiation in HC11 cells.

One of the main requirements for the activation of the β-casein promoter is the interaction of the epithelial cells with the basement membrane, which is composed primarily of laminin. Since CTGF/CCN2 is a matrix-associated protein, its effect on β-casein transcription in the presence of additional matrix proteins was investigated. Previous studies have shown that CTGF/CCN2 acts as a stromal mediator by binding to integrins and other components of the extracellular matrix (84, 111), and the interaction between β1 integrins and matrix components is required for lactogenic differentiation, namely the activation of the prolactin receptor and β-casein transcription (246). Each cell line was therefore seeded on fibronectin, collagen I, or Matrigel coated or uncoated tissue culture dishes. Following the stimulation of the cells with DIP, total RNA was extracted and used to analyze β-casein expression by northern blot. As expected, the results showed a consistently higher level of β-casein mRNA expression in the HC11-TRE-CTGF cells compared with the HC11-TRE vector control cells (**Figure 10a**). The results also indicated a greater than 3-fold increase in β-casein transcription in HC11-TRE cells grown on the various matrices compared to cells grown on plastic. In contrast, HC11-TRE-CTGF cells did not display the same fold increases of β-casein transcription when grown on fibronectin, collagen I or Matrigel. These findings are clearly depicted by looking at the results in terms of fold induction (**Figure 10b**). Thus, it appears that ectopic expression of CTGF/CCN2 not only enhances the level of β-casein transcription in the absence of additional matrix proteins, but it decreases the influence of matrix

components that typically contribute drastically to the initiation of β-casein transcription. Moreover, the results suggest that CTGF/CCN2 contributes significantly to the requirement for laminin-receptor engagement in this system.

Figure 10

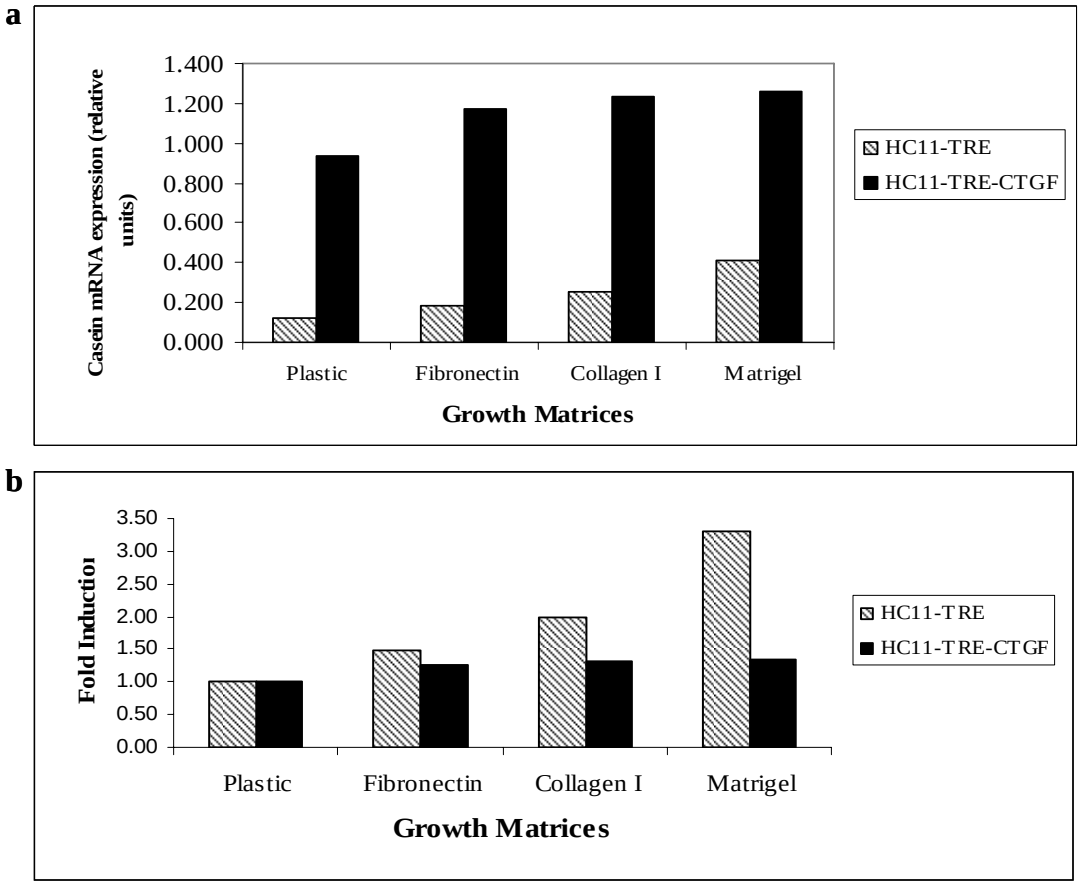


Figure 10. ***CTGF/CCN2 partially abrogates the requirement of matrix proteins for β-casein transcription in HC11 cells***. HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free conditions on respective coated or uncoated tissue culture dishes and once confluent, induced to differentiate with DIP for 48 hours. Total RNA was harvested and levels of β-casein transcript were determined by northern blot analysis and normalized to actin (**a**). Contribution of the matrix proteins to β-casein transcription can be seen by fold induction analysis of the RNA from the northern blot (**b**).

Part 3: Connective Tissue Growth Factor as a Mediator of Cell Survival in Mouse Mammary Epithelial Cells

We have been able to show that CTGF/CCN2 contributes significantly to the generation of signals from the basement membrane to the β-casein promoter. Previously, we have shown that elevated expression of CTGF/CCN2 enhances the expression of β1 integrin as well as the activation of downstream mediator focal adhesion kinase (FAK) (271). Thus, investigation into the effect of CTGF/CCN2 on other integrin-mediated events was warranted.

CTGF/CCN2 is known to be involved in matrix production and proliferation in concert with other growth factors in many cell types (80, 254) and directly involved in cell adhesion and differentiation in a variety of cell types (9, 193). The cell adhesion and differentiation effects mediated by CTGF/CCN2 are commonly found to be the result of the interaction between CTGF/CCN2 and integrin complexes (7, 34, 35, 85, 87). Upon the genetic deletion of β1 integrin, mammary epithelial cells undergo cell cycle arrest and the glands display defective development *in vivo*, suggesting that mammary epithelial cell growth and survival requires integrin-mediated adhesion (186). The data presented in the proceeding sections will thus address the possible effect of CTGF/CCN2 on the integrin-mediated adhesion and survival of mammary epithelial cells.

CTGF/CCN2 contributes to cell proliferation in HC11 cells

To determine if the contribution of CTGF/CCN2 extends beyond the enhancement of lactogenic differentiation to the growth and survival of HC11 cells, CTGF/CCN2-expressing HC11 cells were compared to vector controls for proliferative

capacity, or cell viabilty. The HC11-TRE-CTGF and HC11-TRE cells were grown in serum-free media with EGF, and viability was determined at 24, 48, 72, and 96 hours by MTT assay (**Figure 11**). The MTT assay specifically detects cells that are metabolically viable through their ability to convert the MTT dye into a water-soluble formazan salt, which is solubilized and quantified by reading the absorbance at 570nm. Enhanced absorbance is indicative of a higher number of viable cells, and therefore the proliferative capacity of the cells in the well. The results indicated that HC11-TRE-CTGF cells displayed significantly more proliferation than the HC11-TRE cells. HC11-TRE cells grew until 48 or 72 hours, after which time they ceased significant enhancement of proliferation. The proliferative capacity was enhanced and sustained by CTGF/CCN2 expression at later times when HC11-TRE cells showed no further increases in proliferation.

Because CTGF/CCN2 is a secreted protein, conditioned media from HC11-TRE-CTGF cells was also employed to determine the effect of CTGF/CCN2 on the growth of HC11 cells. The same 38kd protein found in cell lysates is also present in the media. Results, shown in **Figure 12**, indicated that CTGF/CCN2-conditioned media sustained the growth of control cells through 96 hours compared to the control media. Again, the most significant differences were observed at later time points. This suggested that CTGF/CCN2 may play a role in the survival of cells under serum-free conditions.

Figure 11

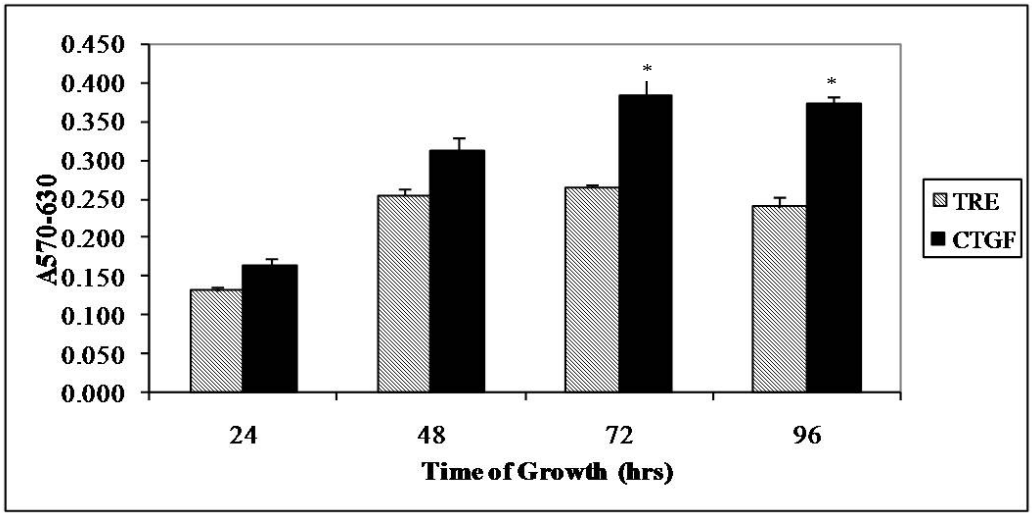


Figure 11. ***CTGF/CCN2 expression sustains the proliferation of HC11 cells.*** HC11-TRE and HC11-TRE-CTGF cells were seeded in 96-well microtiter plates in serum-free media with EGF (10ng/ml). Cell proliferation was determined at 24, 48, 72, and 96 hours post addition of EGF using the MTT assay. The results are reported as the mean $\pm$ S.D. of four determinations. *, $p < 0.005$.

Figure 12

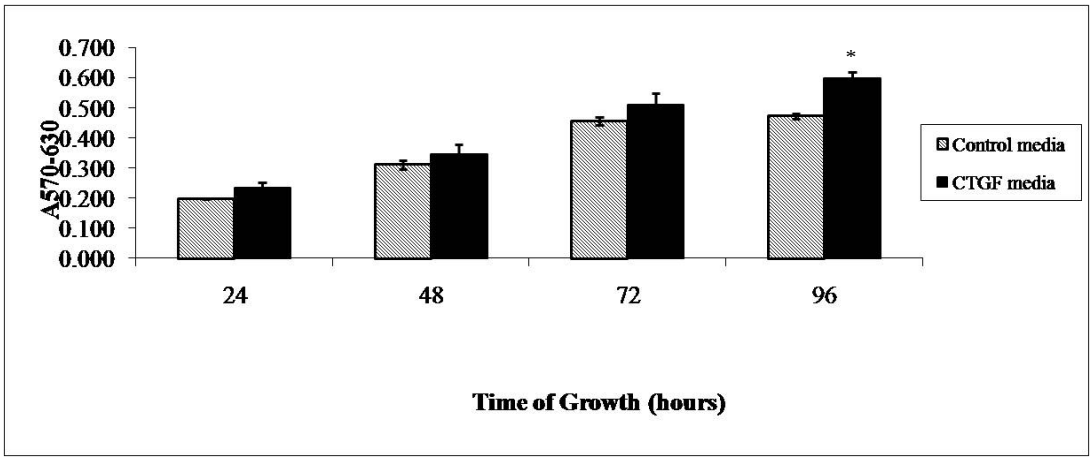


Figure 12. ***Secreted CTGF/CCN2 sustains the proliferation of HC11 cells.*** HC11 cells were seeded in control serum-free media with EGF (10ng/ml) plus conditioned media from HC11-TRE-CTGF cells that had previously been grown in serum-free media. Cell proliferation was determined at 24, 48, 72, and 96 hours post addition of EGF using the MTT assay. The results are reported as the mean $\pm$ the S.D. of four determinations. *, $p < 0.005$.

CTGF/CCN2 mediates cell proliferation and survival through integrins

In order to determine whether the CTGF/CCN2-mediated effects on cell proliferation and survival are dependent on the interaction between CTGF/CCN2 and integrin complexes, an MTT assay was performed in the presence of an integrin binding RGD-containing peptide. RGD-containing peptides bind to the site on integrin molecules that is responsible for binding to matrix proteins, such as laminin, and therefore acts as a competitive inhibitor to other proteins with binding affinity for the integrin (222).

HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free conditions in the presence of EGF (10ng/ml) with or without the RGD-containing peptide (500μM), or a control RAD-containing peptide (500μM). Levels of proliferation were determined at 24, 48, 72, and 96 hours by MTT assay. Results, shown in **Figure 13**, display a normal serum-free proliferation curve for the HC11-TRE control cells and an expected enhanced proliferation of the HC11-TRE-CTGF cells through 96 hours. The HC11-TRE-CTGF cells incubated with the RGD-containing peptide show less proliferative capabilities than the cells incubated with the RAD-containing control peptide or the HC11-TRE-CTGF cells grown in the absence of any peptide, especially at the later time points. These results suggest that the RGD-containing peptide is partially blocking the ability of CTGF/CCN2 to bind to integrins, thereby decreasing the effect of CTGF/CCN2 on the enhanced viability of the HC11 cells under serum-free conditions. Together with the results from the first two MTT analyses, these results confirm that CTGF/CCN2 enhances cell proliferation through an interaction with integrin complexes.

Figure 13

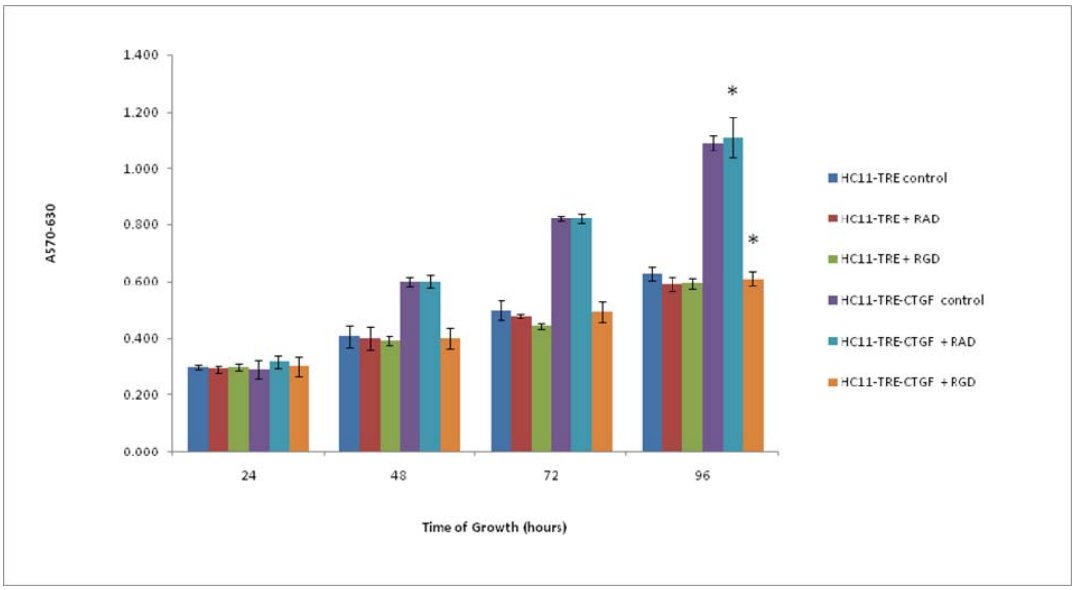


Figure 13. ***CTGF/CCN2-mediated survival is dependent on integrins.*** In order to determine whether the effects-mediated by CTGF/CCN2 are dependent on integrin complexes, HC11-TRE and HC11-TRE-CTGF cells were incubated with control serum-free media with EGF (10ng/ml), or serum-free media containing either an RGD-containing peptide (500μM) or RAD-containing peptide, where indicated, with EGF (10ng/ml). The MTT assay was performed at 24, 48, 72, and 96 hours. The results are displayed as the mean $\pm$ the S.D. of four determinations. *, $p < 0.005$.

CTGF/CCN2 facilitates movement of HC11 cells through the cell cycle

The ability of CTGF/CCN2 to maintain the proliferative capacity of the cells under serum-free conditions has been shown. In order to determine whether the effect of CTGF/CCN2 is specific for proliferation or also applies to cell survival, a cell cycle analysis was performed. To determine the effect of CTGF/CCN2 expression on the ability of HC11 cells to move through the cell cycle, HC11-TRE and HC11-TRE-CTGF cells were harvested after 96 hours, or 4 days, of being maintained under serum-free conditions and subjected to staining with propidium iodide (PI) and analysis by flow cytometry. Data shown in **Figure 14a** shows the percentage of total HC11-TRE and HC11-TRE-CTGF cell populations in sub G_0/G_1, G_1, S, and G_2/M phases of the cell cycle at day 4. **Figure 14b** shows a graphical cell cycle analysis comparison of the HC11-TRE and HC11-TRE-CTGF cell lines at day 4. In each cell line the majority of cells were in the G_1 phase of the cell cycle. However, the results indicated that cells with enhanced expression of CTGF/CCN2 had greater populations in both S and G_2/M phases, while HC11-TRE cells showed a greater population of cells in sub G_0/G_1, which is indicative of possible apoptosis.

Figure 14

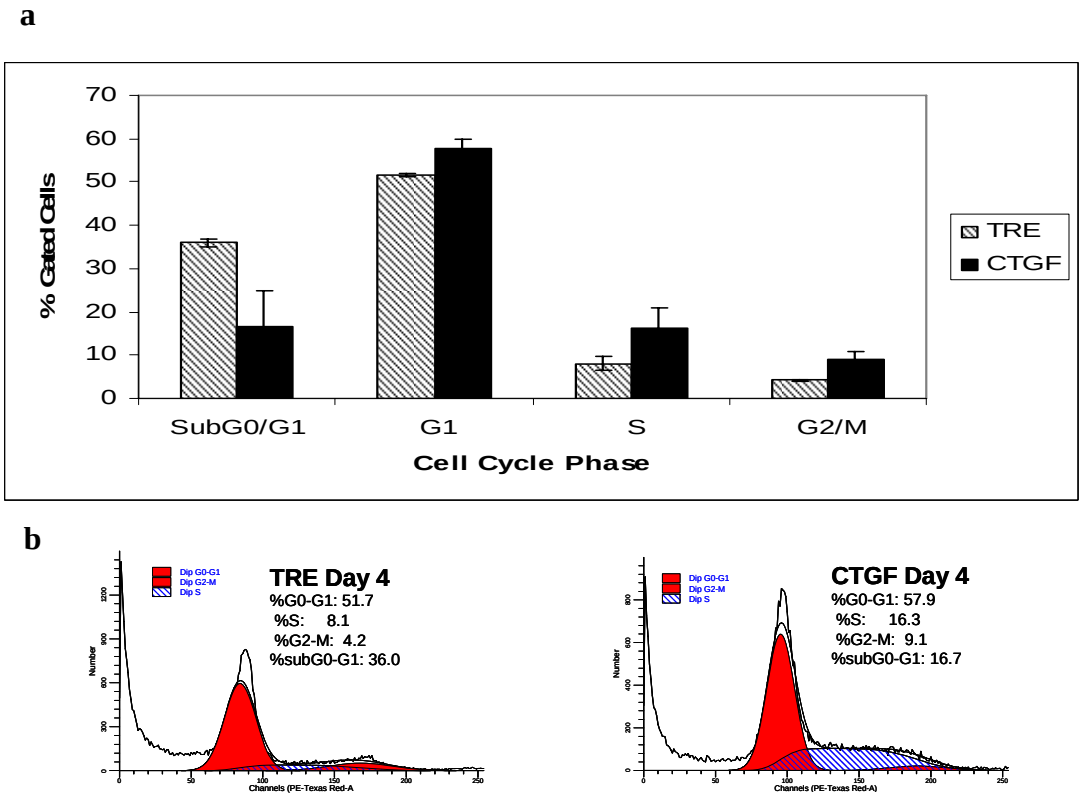


Figure 14. ***CTGF/CCN2 contributes to cell cycle progression in HC11 cells.*** HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free media in the presence of EGF (10ng/ml) and harvested after 96 hours. Cells were fixed in ethanol and stained with propidium iodide (50µg/ml) prior to cell cycle analysis by flow cytometry. Data is representative of three determinations. (**a**) Percentage of total gated cells in each phase of the cell cycle. (**b**) Graphical analysis of cell cycle distribution by DNA content.

CTGF/CCN2 inhibits anoikis in HC11 Cells

To confirm apoptosis, a TUNEL assay and PARP cleavage analysis were performed. In the absence of serum, adherent epithelial cells typically detach and set off an apoptotic response referred to as anoikis. When epithelial cells lose their integrin-mediated attachment, apoptosis is initiated due to the lack of survival signaling, which leads to an imbalance in the ratio of anti-apoptotic factors to pro-apoptotic factors (210). Pro-apoptotic factors such as Bax or Bid form homodimers in the mitochondrial membrane leading to the release of cytochrome C and subsequent activation of the caspase cascade (91). Activation of this cascade leads to multiple events including the fragmentation of DNA. Thus, to determine the level of possible apoptosis, the cells were seeded in serum-free media for 96 hours, fixed, and examined for TUNEL, a measure of DNA fragmentation. The results, shown in **Figure 15**, indicated a higher level of TUNEL staining in HC11-TRE cells compared to the HC11-TRE-CTGF cells. To further support this data, cells were grown in serum-free media in the presence of EGF and at 96 hours protein lysates were collected to determine the levels of total and cleaved PARP by western blotting. PARP cleavage is a common marker of the activation of a caspase-dependent apoptotic pathway (56). Results, shown in **Figure 16**, indicated that HC11-TRE cells displayed greater PARP cleavage compared to the HC11-TRE-CTGF cells after 4 days of being maintained in the absence of serum. In conclusion, these results demonstrated that CTGF/CCN2 contributes to the survival of HC11 cells.

Figure 15

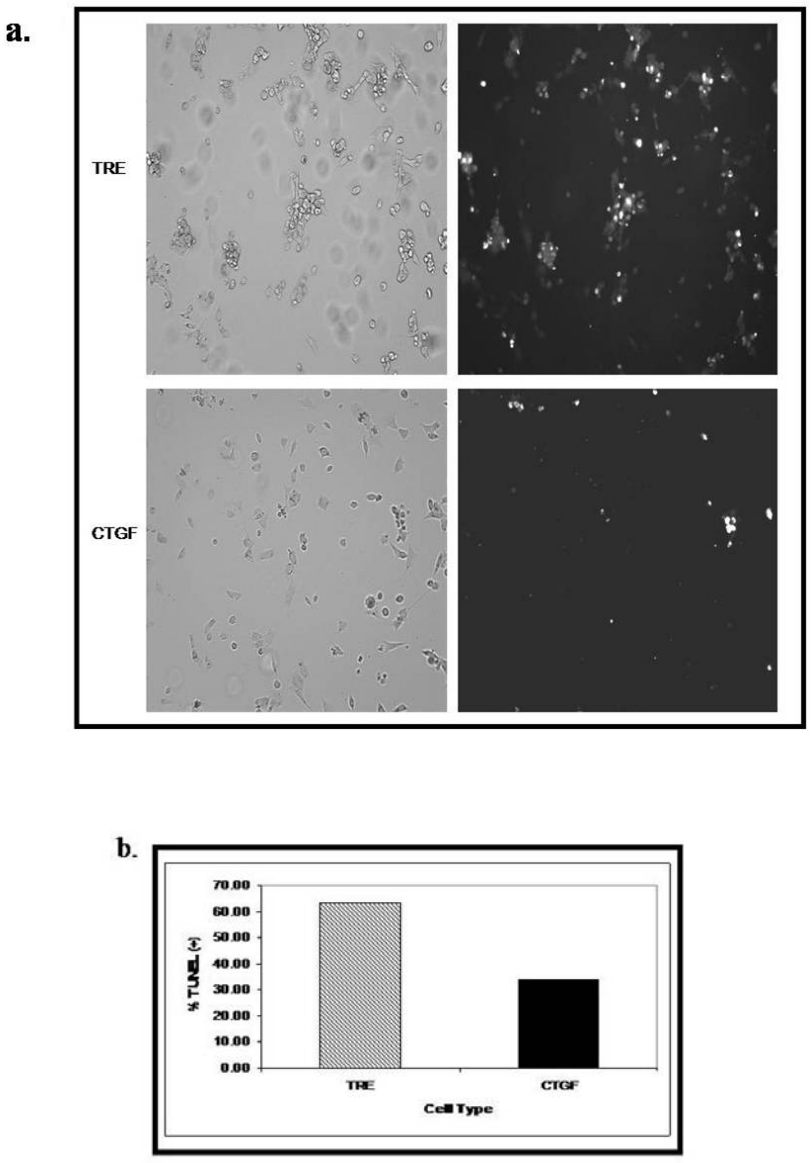


Figure 15. ***CTGF/CCN2 protects cells from anoikis***. HC11-TRE and HC11-TRE-CTGF cells were seeded on coverslips in serum-free media in the presence of EGF (10ng/ml) for 96 hours, at which time they were fixed and apoptosis was detected by TUNEL. (**a**) Left panels depict cell morphology, while the panels on the right display positive TUNEL staining in white. (**b**) Quantitation of TUNEL staining.

Figure 16

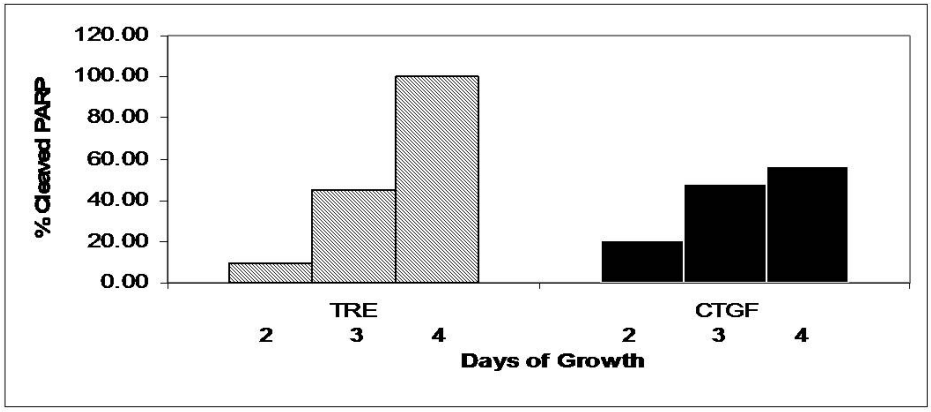


Figure 16*. *CTGF/CCN2 expression results in reduced PARP cleavage. HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free media in the presence of EGF (10ng/ml) and harvested at 2, 3, and 4 days. PARP cleavage was detected by western blot. Shown is the quantitation of % PARP cleavage, determined by densitometry.

CTGF/CCN2 activates survival signaling pathways in HC11 cells.

Upon the genetic deletion of β1 integrin, mammary glands display defective development *in vivo*, suggesting that mammary epithelial cell growth and survival requires integrin-mediated adhesion (186). To elucidate the mechanism by which CTGF/CCN2 regulates survival, studies were performed to determine the contribution of CTGF/CCN2 to the activation of β1 integrin-mediated survival signaling. Recent literature from our lab and others suggests that CTGF/CCN2 binds to and enhances the expression of β1 integrin (87, 271). Protein lysates from HC11-TRE-CTGF and HC11-TRE cells were collected after 96 hours of cell growth in serum-free conditions and used to determine the expression of total and activated mediators of β1 integrin adhesion and survival signaling (**Figure 17**). The results indicated that HC11-TRE-CTGF cells display enhanced expression of β1 integrin, phosphorylated FAK, phosphorylated Akt, and cyclin D1 compared to expression in HC11-TRE cells.

Figure 17

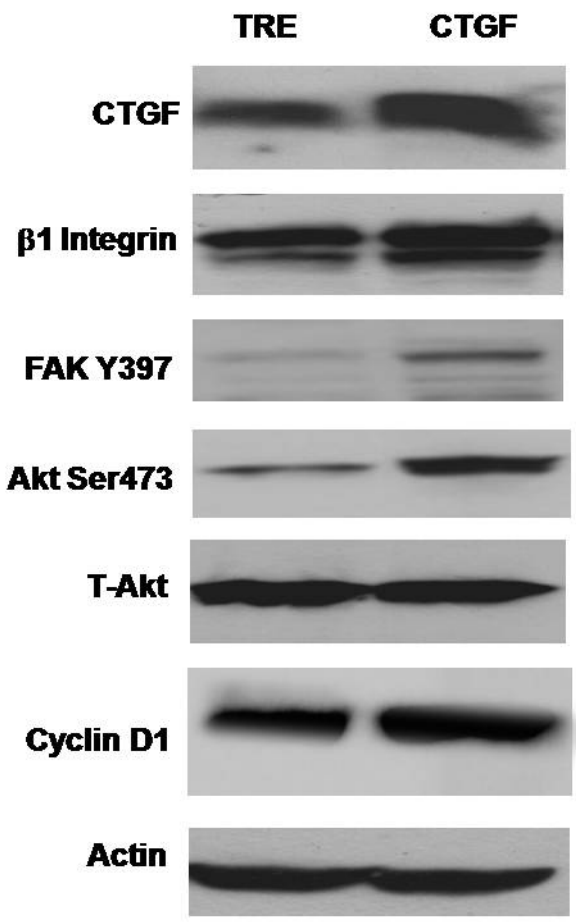


Figure 17. ***CTGF/CCN2 contributes to the activation of survival protein downstream of β1 integrin.*** HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free media with EGF (10ng/ml). Lysates were harvested for protein after 96 hours and used to determine the expression levels of the indicated survival proteins by western blot. Results are representative of 3 experiments.

CTGF/CCN2 contributes to the formation of focal adhesion complexes in HC11 cells

Interactions between mammary epithelial cells and the basement membrane are crucial to cell survival. To further understand the mechanism by which CTGF/CCN2 enhances survival signaling, the contribution of CTGF/CCN2 to the formation of focal adhesion complexes was explored. HC11-TRE and HC11-TRE-CTGF cells were grown for 96 hours in serum-free media in the presence of EGF. Cells were harvested and protein lysates were used to determine the expression levels of focal adhesion proteins by western blot (**Figure 18**). Results revealed increases in the expression levels of adaptor proteins paxillin, parvin, and p130cas, as well as structural proteins vinculin, PINCH, and Rsu-1 in HC11-TRE-CTGF cells compared to the levels seen in control HC11-TRE cells. Furthermore, HC11-TRE-CTGF cells displayed increases in the kinases Src and ILK. Results are supported by clear visualization of a greater number of focal adhesions formed in HC11-TRE-CTGF cells compared to vector control HC11-TRE cells as seen by immunofluorescent staining of vinculin in **Figure 19**. In addition, the focal adhesions in HC11-TRE-CTGF cells are larger. In order to visualize clear formation of the focal adhesions, the cells were seeded in 0.5% serum-containing media.

Figure 18

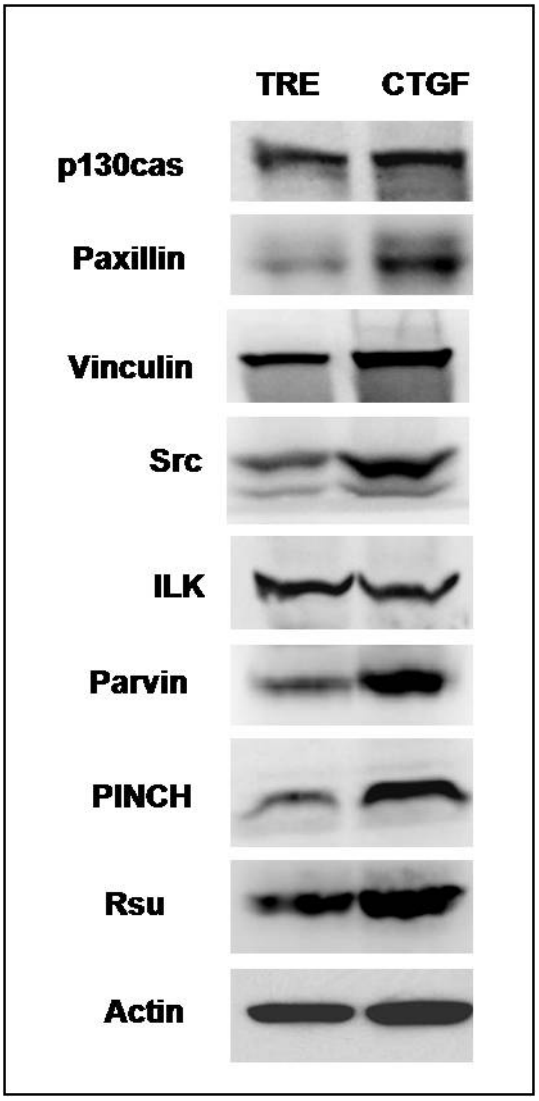


Figure 18. ***CTGF/CCN2 enhances the levels of focal adhesion complex proteins.*** HC11-TRE and HC11-TRE-CTGF cells were seeded in serum-free media plus EGF (10ng/ml). Protein lysates harvested at 96 hours were used to detect levels of focal adhesion-related proteins by western blot. The data shown is representative of three independent experiments.

Figure 19

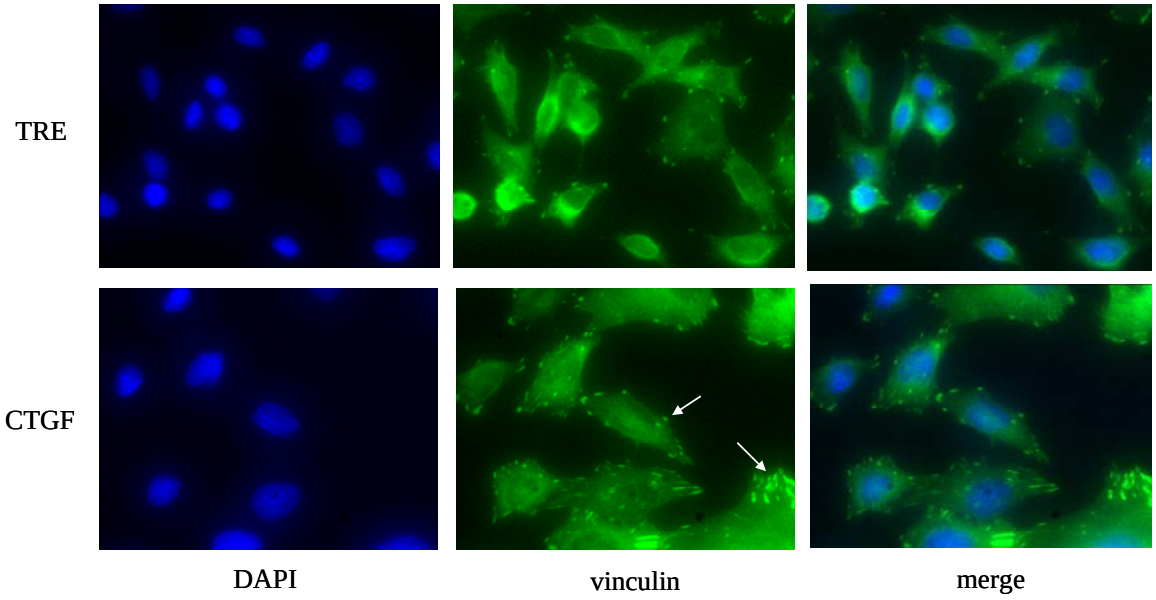


Figure 19. ***Focal adhesion formation is enhanced in cells with enhanced expression of CTGF/CCN2.*** HC11-TRE and HC11-TRE-CTGF cells were seeded in 0.5% serum-containing media with EGF (10ng/ml) for 4 days. The cells were then fixed in paraformaldehyde and stained for the presence of vinculin (green) with a DAPI nuclear counterstain. Focal adhesions are shown as punctate bodies primarily near the edges of cells. Magnification = 40X.

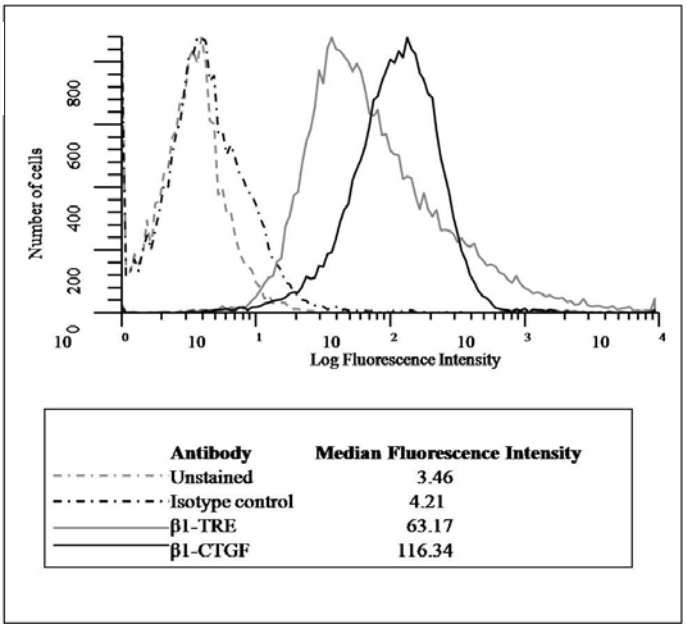
CTGF/CCN2 enhances surface level expression of both α6 and β1 integrins

The most predominant protein of the basement membrane is laminin, and the interaction between β1 integrin complexes and laminin is required for both the survival of the mammary epithelial cells as well as for lactogenic differentiation (75). A common receptor for laminin is the α6β1 integrin complex (123), and CTGF/CCN2 is known to bind to this complex in various cell types (154, 255). This receptor is critical not only for

lactogenic differentiation, but also for mammary gland development in general. Thus far our results have shown that CTGF/CCN2 enhances the expression of surface-level α6 and β1 integrins and the formation of focal adhesion complexes. Results, shown in **Figure 2,** indicated that HC11-TRE-CTGF cells display a greater level of both α6 and β1 integrin on their surface compared to the HC11-TRE control cells. Together with the effect of CTGF/CCN2 on survival signaling pathways, these results suggest that CTGF/CCN2 may mediate survival signaling through α6β1 integrin complex stabilization and activation.

Figure 20

a



b

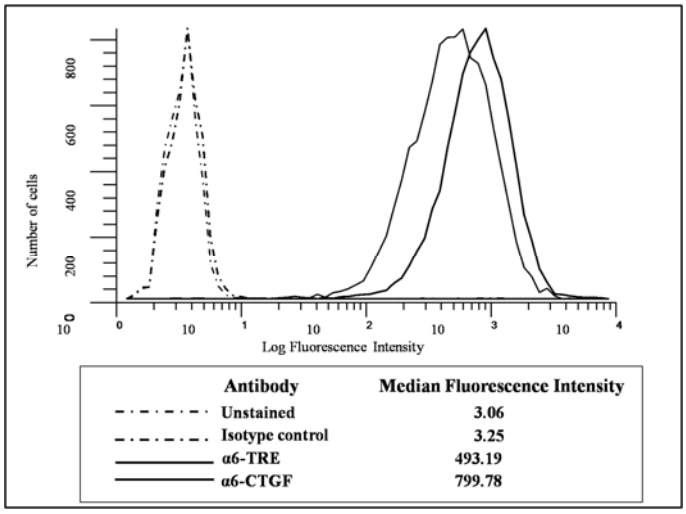


Figure 20. ***CTGF/CCN2 contributes to surface level expression of α6 and β1 integrins.*** HC11-TRE and HC11-TRE-CTGF cells were grown in the absence of serum for 4 days in the presence of EGF (10ng/ml). Cells were harvested and stained with an anti-β1 integrin antibody (**a**) or an anti-α6 antibody (**b**) followed by detection with a fluorescence-conjugated secondary and analysis by flow cytometry. The median number of β1 integrin or α6 positive cells are indicated in the tables. The data are representative of 4 independent experiments.

CTGF/CCN2 enhances cell adhesion via the α6β1 integrin complex

In order to finally determine whether CTGF/CCN2 was acting to mediate cell adhesion and not just the expression of adhesion-related proteins, assays were performed where HC11-TRE control cells were allowed to attach to either control or CTGF/CCN2-coated plates.

HC11-TRE control cells were allowed to attach to recombinant CTGF/CCN2-coated microtiter wells blocked with 1% BSA for 4 hours. Adherent cells were fixed and stained with 0.1% crystal violet and the absorbance at 570nm was determined for quantitation. Results revealed a significant increase in the number of adherent cells seeded on CTGF/CCN2-coated wells compared with those seeded on the BSA-coated control wells (**Figure 21**). This enhanced adhesion was blocked in the presence of EDTA, and rescued with the addition of the divalent cation Mg^{2+}. Because EDTA is known to inhibit integrin-mediated attachments (154), these results suggest that the enhanced adhesion of the HC11-TRE cells to the CTGF/CCN2 is likely to be mediated by integrins.

To confirm that the integrin complex involved in the CTGF/CCN2-mediated enhancement of adhesion is the α6β1 complex, the same adhesion assay was performed in the presence of function blocking antibodies against both α6 and β1 integrins, as well as function blocking antibodies against αv and β3 integrins (**Figure 22**). The addition of only the anti-α6 or anti-β1 antibodies was able to inhibit the CTGF/CCN2-mediated increase in adhesion, while the addition of the anti-αv, anti-β3, or isotype control antibodies showed no significant change in CTGF/CCN2-mediated adhesion or the adhesion levels on the control BSA-coated plates. Together with the flow cytometry data

showing CTGF/CCN2-mediated enhancement of surface level expression of the α6β1 integrin complex, these results confirm the involvement of the α6β1 integrin complex in the CTGF/CCN2-mediated increase in HC11 cell adhesion.

Figure 21

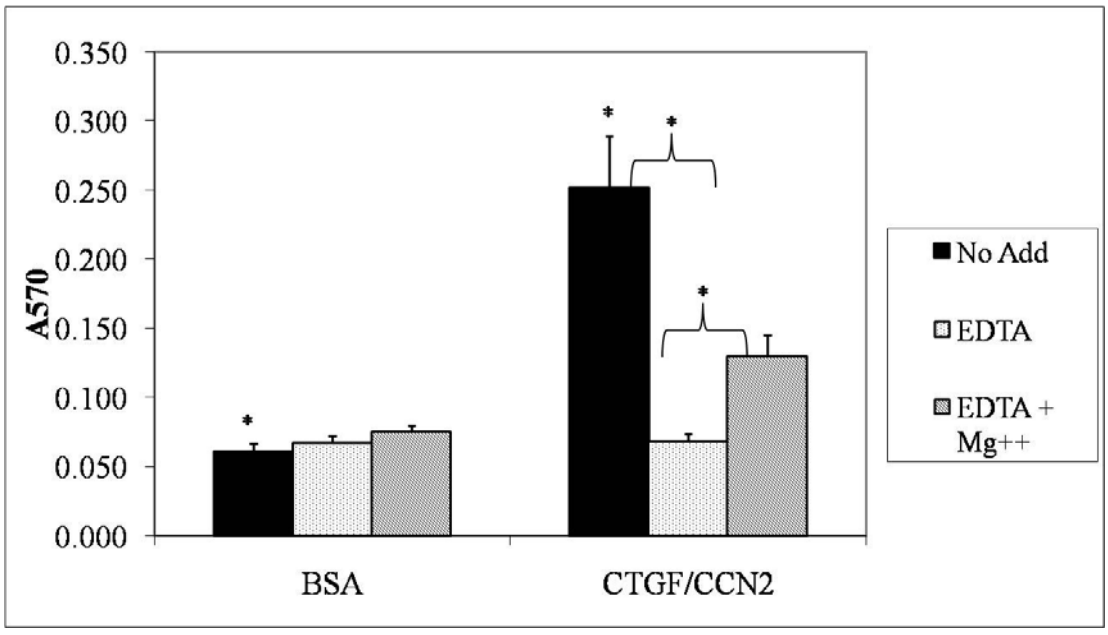


Figure 21. *HC11* ***cells display enhanced adhesion to CTGF/CCN2***. Maleic anhydride Reacti-Bind microtiter wells were coated with recombinant CTGF/CCN2 (2μg/ml) or 1% BSA overnight at 4°C and blocked with 1% BSA. HC11-TRE cells were resuspended in serum-free growth media + EGF (10ng/ml) and, where indicated, EDTA (2.5mM) or EDTA + Mg^{2+} (5mM) and seeded on coated wells. The cells were allowed to adhere for 4 hours at 37°C. Adherent cells were fixed with 3.7% formaldehyde, stained with 0.1% crystal violet, and quantified by reading the absorbance at 570nm. Data are means of quadruplicate samples, error bars represent standard error. *, $p < 0.005$.

Figure 22

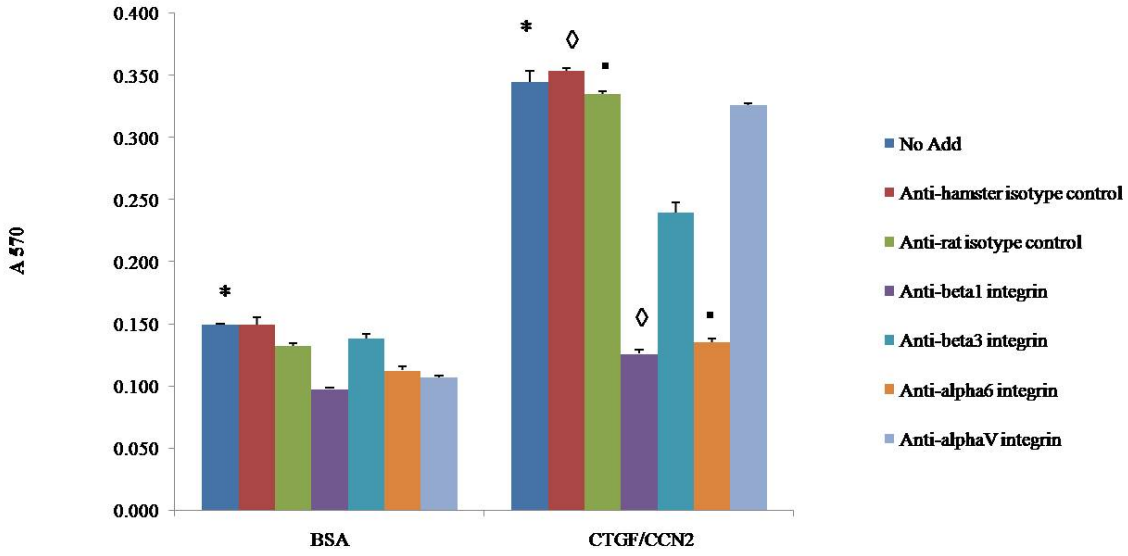


Figure 22. ***HC11 cells display enhanced adhesion to CTGF/CCN2 via the α6β1 integrin complex.*** Maleic anhydride Reacti-Bind microtiter wells were coated with recombinant CTGF/CCN2 (2μg/ml) or 1% BSA overnight at 4°C and blocked with 1% BSA. HC11-TRE cells were resuspended in serum-free growth media + EGF (10ng/ml) and, where indicated, function-blocking antibodies (25μg/ml), and seeded on coated wells. The cells were allowed to adhere for 4 hours at 37°C. Adherent cells were fixed with 3.7% formaldehyde, stained with 0.1% crystal violet, and quantified by reading the absorbance at 570nm. Data are means of quadruplicate samples, error bars represent standard error. *, ◊, ▪, $p < 0.005$.

CTGF/CCN2 contributes to the transcriptional regulation of growth factor-, cell cycle-, extracellular matrix-, development-, and survival-associated genes in HC11 cells.

In a study by Kennedy *et al*., CTGF/CCN2-null mouse embryonic fibroblasts (MEFs) were isolated for genome-wide mRNA expression profiling analysis (132). It was determined from this study that *ctgf*/*ccn2*-/- mice displayed significantly reduced expression of pro-adhesive, pro-inflammatory and pro-angiogenic genes (132). The findings from *ctgf*/*ccn2*-/- fibroblasts support the discoveries in HC11 cells suggesting that CTGF/CCN2 contributes to adhesion and survival.

To confirm the role of CTGF/CCN2 in HC11 cells, a genome-wide mRNA expression analysis, followed by a gene ontology analysis, was done on the HC11-TRE and HC11-TRE-CTGF cells grown under normal conditions. For general microarray analysis, technical replicates were combined and a student's *t*-test was used to establish p-values. Fold changes were calculated based on the means of the values of the technical replicates for each probe. For the gene ontology analysis, a set of "affected genes" was established using a p-value requirement of 0.05 and a two-fold change minimum. It was determined by the gene ontology analysis (**Table 1**) that HC11-TRE-CTGF cells express significantly elevated levels of extracellular region-associated and development-associated genes, as well as higher levels of growth factor-associated, cell cycle and survival promoting, and structural morphogenesis-related genes compared with the HC11-TRE control cells. Also in support of CTGF/CCN2 as a pro-survival factor, a decreased level of pro-apoptotic genes was evident in the HC11-TRE-CTGF cells compared with the HC11-TRE vector control cells.

To establish a valid comparison of the role of CTGF/CCN2 as a pro-adhesion and pro-survival factor in both fibroblasts and HC11 cells, **Table 2** shows a set of genes that were found to be both downregulated in *ctgf/ccn2-/-* fibroblasts and upregulated in HC11-TRE-CTGF cells. The genes were primarily adhesion-related, such as laminin and fibronectin, and cadherins.. The results from the microarray analysis confirm the role of CTGF/CCN2 as a regulator of epithelial cell adhesion and survival in HC11 mouse mammary epithelial cells.

Table 1. Functional division of genes transcriptionally regulated in HC11-TRE-CTGF cells

Up-Regulated Genes	Mean of log ratio
Extracellular matrix signaling-associated	
Wingless-type MMTV integration site family 4, Wnt 4	3.18
Artemin	2.17
Tissue inhibitor of metalloproteinase, TIMP3	2.04
Tumor necrosis factor, alpha-induced protein 6, TNFaip6	1.96
Inhibin, beta A	1.81
Fibronectin 1, Fn1	1.76
ADAM metallopeptidase with thrombospondin 1 motif, ADAMTS-1	1.62
Protein tyrosine phosphatase, receptor type K, ptprk	1.61
Laminin, beta2 subunit	1.6
Plasminogen activator, urokinase receptor, Plaur	1.58
ADAMTS-like 4	1.55
Thrombospondin 1, TSP1	1.43
Laminin, alpha5 subunit	1.4
Survival, Cell Cycle-associated	
Dual specificity protein phosphatase 8, Dusp8	4.1
Dual specificity protein phosphatase 1, Dusp1, Mkp1	3.85
Dual specificity protein phosphatase 3, Dusp3	2.78
Dual specificity protein phosphatase 16, Dusp16, Mkp7	2.47
Epiregulin	2.39
TNF receptor-associated factor 1, Traf1	2.39
Myc	2.3
Ras homolog gene family, member U, RhoU	1.84
Inhibin, beta A	1.81
ADAMTS-1	1.62
Myeloid leukemia factor 1, Mlf1	1.57
ADAMTS-l4	1.55
E2F transcription factor, E2F2	1.44
Tensin 4	-1.5
BCL2/adenovirus E1B interacting protein 3, Nip3	-1.54
Death-associated protein	-1.83
Caspase 6	-2.57
Egl nine homolog 1, Egln	-3.94

Development-associated	
Wingless-type MMTV integration site family 4, Wnt 4	3.18
Epigen	2.91
Ets domain transcription factor, epithelial specific, Elf3	2.43
TNF receptor-associated factor 1, Traf1	2.39
Myc	2.3
Artemin	2.17
Tissue inhibitor of metalloproteinase, TIMP3	2.04
v-rel reticuloendotheliosis viral oncogenes homolog B, relB	1.92
Tumor necrosis factor receptor superfamily 12a, Tnfrsf12a	1.92
Ras homolog gene family, member U, RhoU	1.84
Inhibin, beta A	1.81
Fibronectin 1, Fn1	1.76
Jun D proto-oncogene, JunD	1.63
ADAMTS-1	1.62
Plasminogen activator, urokinase receptor, Plaur	1.58
Myeloid leukemia factor 1, Mlf1	1.57
ADAMTS-l4	1.55
Ras homolog gene family, member B, RhoB	1.48
Cell Differentiation-associated	
Wingless-type MMTV integration site family 4, Wnt 4	3.18
Ets domain transcription factor, epithelial specific, Elf3	2.43
Epiregulin	2.39
v-rel reticuloendotheliosis viral oncogenes homolog B, relB	1.92
Myeloid leukemia factor 1, Mlf1	1.57
Growth Factor, Catalytic Activity-associated	
Dual specificity protein phosphatase 8, Dusp8	4.1
Dual specificity protein phosphatase 1, Dusp 1, Mkp1	3.85
Fibroblast growth factor 13, Fgf13	3.54
Polo-like kinase 3, Plk3	3.27
Epigen	2.91
Amphiregulin	2.85
Dual specificity protein phosphatase 3, Dusp3	2.78
Dual specificity protein phosphatase 16, Dusp16, Mkp7	2.47
Ras homolog gene family, member D, RhoD	2.28
Artemin	2.17
Diaphanous homolog 3, Diaph3	1.94
Ras homolog gene family, member U, RhoU	1.84

Inhibin, beta A	1.81
Fibronectin 1, Fn1	1.76
Thyroid hormone receptor interactor 6, Trip6	1.68
ADAMTS-1	1.62
Protein tyrosine phosphatase, receptor type K, ptprk	1.61
ADAMTS-l4	1.55
Structural Morphology-associated	
Wingless-type MMTV integration site family 4, Wnt 4	3.18
Ets domain transcription factor, epithelial specific, Elf3	2.43
Epiregulin	2.39
Myc	2.3
Tissue inhibitor of metalloproteinase, TIMP3	2.04
Fibronectin 1, Fn1	1.76
Plasminogen activator, urokinase receptor, Plaur	1.58

The functional division of genes transcriptionally regulated by CTGF/CCN2 in HC11 cells. The list does not include all genes with 2-fold or greater increase. Some genes are listed in more than one category.

Table 2. Genes transcriptionally regulated by CTGF/CCN2 in MEFs and HC11 cells.

Gene name	**MEFs (*ccn2*-/-)**	**HC11-TRE-CTGF**
Laminin α5	↓	↑
Laminin β2	↓	↑
T-cadherin	↓	↑
LI-cadherin	↓	↑
Thrombospondin 1	↓	↑
Syndecan 4	↓	↑
Fibronectin	↓	↑

A comparison of the genes transcriptionally regulated by CTGF/CCN2 in HC11 cells and mouse embryonic fibroblast (MEFs) (132). The list does not include all genes with 2-fold or greater increase in either cell line. ↓, downregulation; ↑, upregulation.

Chapter 4: Discussion

Introduction

The mammary gland constantly undergoes cycles of development and differentiation. Mammary epithelial cells, in particular, undergo cyclical phases of proliferation, differentiation and cell death throughout the lifespan of the gland. The mechanisms used by the cells during these cycles require the strict regulation of growth factor- and extracellular matrix (ECM) interaction - mediated signal transduction pathways. Many of these pathways can be manipulated by abnormal cells that may be present in the mammary gland in order to support their growth and survival. Hence, the delineation of the mechanisms involved in the growth, differentiation, and survival of mammary epithelial cells is critical to our understanding of the development of abnormalities of the breast.

Of the cycles that mammary epithelial cells undergo throughout development, this study focuses primarily on the mechanisms involved during lactogenic differentiation, which is a complex process involving lactogenic hormone- and ECM-mediated events. In response to these signaling events, the secretory cells produce milk proteins, with β-casein being the predominant milk protein used as a marker to determine the level of lactogenic differentiation in mammary epithelial cells. Elucidation of the mechanisms critical for activating the transcription of β-casein will advance our understanding of the mechanisms required for the lactogenic differentiation of mammary epithelial cells.

Many studies have addressed the involvement of the lactogenic hormone prolactin in the activation of β-casein transcription (11, 13, 24, 131, 189, 200). Fewer studies have

described the exact contribution of the ECM to the activation of β-casein transcription (3, 33, 62, 75), yet it has been determined that the interaction of the epithelial cells with the ECM is required for the activation of the β-casein promoter (125, 185, 219). Previously, our lab determined that Connective Tissue Growth Factor (CTGF/CCN2), a known stromal mediator, is required for the transcription of β-casein in mouse mammary epithelial cells (271), yet the mechanism of CTGF/CCN2 was yet undetermined.

This thesis presents data supporting my overall hypothesis that CTGF/CCN2 acts as an adhesion and survival factor for mouse mammary epithelial cells via the stabilization of β1 integrin-containing complexes and subsequent enhancement of their downstream signaling pathways, including the activation of β-casein transcription. I have presented data supporting the hypothesis that the role of CTGF/CCN2 in lactogenic differentiation is a contribution to the activation of the prolactin receptor signaling pathway and ultimately the binding of Stat5 to the β-casein promoter. I also present data supporting the hypothesis that CTGF/CCN2 functions to stabilize β1 integrin-mediated adhesion and resulting survival of mammary epithelial cells. Ultimately, the CTGF/CCN2-mediated stabilization of the adhesive properties of the mammary epithelial cells enhances the activation of the β-casein promoter and subsequent transcription of the milk protein when the cells are stimulated to undergo lactogenic differentiation, which affirms the central hypothesis of this thesis.

The Contribution of CTGF/CCN2 to the Activation of β-casein Transcription

In order for mammary epithelial cells to undergo lactogenic differentiation, they must undergo a shift in their transcriptional pattern from the support of proliferation to

the support of differentiation functions. One of the major components of this change in mammary epithelial cells is the regulation of genes that enhance the production of ECM proteins (271), and this is transcriptionally regulated, in part, by glucocorticoids. Previously, data published from our lab showed that glucocorticoid-mediated CTGF/CCN2 expression is both elevated and required for HC11 mouse mammary epithelial cell lactogenic differentiation (271). TGFβ is the primary regulator of CTGF/CCN2 expression and function in other cells, such as fibroblasts (235), chondrocytes (21) , and mesangial cells (39), where the deposition of ECM proteins is a primary function. However, we found that the activity of TGFβ is not required for CTGF/CCN2 expression in the HC11 cell background (271). In HC11 cells, CTGF/CCN2 expression can also be regulated by dexamethasone (271). This finding is supported by the discovery of a dexamethasone responsive element in the promoter of CTGF/CCN2 (197). The discovery of the requirement for dexamethasone-mediated CTGF/CCN2 expression in lactogenic differentiation, specifically β-casein transcription, lead to further investigation into the specific mechanism of CTGF/CCN2 in lactogenic differentiation of mouse mammary epithelial cells.

Ectopic CTGF/CCN2 expression enhances β-casein transcription in both HC11 and primary mouse mammary epithelial cell cultures

HC11 mouse mammary epithelial cells are commonly used to study lactogenic differentiation due to their ability to respond to lactogenic hormones in a way that mimics that of primary mouse mammary epithelial cells, namely by the synthesis of β-casein (53). *In vivo,* HC11 cells have the ability to differentiate into ductal structures when

injected into the mammary fat pads of mice (114), thus making them a good model in which to study the mechanisms of lactogenic differentiation. The HC11 cell background was used in this investigation to determine the mechanism of CTGF/CCN2 during lactogenic differentiation.

Previous data from our lab has shown that HC11 cells infected with an adenoviral vector encoding CTGF/CCN2 display a higher level of β-casein transcript compared with HC11 cells infected with a vector control (271). Data presented in this thesis (Figure 4) shows that elevated expression of CTGF/CCN2 in HC11 cells enhances the early transcription of β-casein. Rather than adenoviral infection of HC11 cells, our current studies employ the use of a Tet-off HC11 cell system, such that the removal of doxycycline from culture medium allows for the expression of a CTGF/CCN2-ecoded vector. Ultimately this process results in a stable cell line that can be induced to overexpress CTGF/CCN2. The use of this method may prevent possible damage to the cell or side effects caused by infection with an adenovirus.

Our previous studies were performed on HC11 cells that had been stimulated with lactogenic hormones for 48 hours. We have since learned that the dexamethasone-mediated enhancement of CTGF/CCN2 is much more rapid. Thus, in order to see the true effect of CTGF/CCN2 on the transcription of β-casein, as opposed to a general dexamethasone-induced response, earlier time points after stimulation with lactogenic hormones were assessed. Our results (Figure 4) show that elevated expression of CTGF/CCN2 enhances β-casein transcription at 8 hours, while the vector control cells did not show significant transcription of β-casein until 16 hours post-induction. Surprisingly, there is also expression of β-casein mRNA in the absence of any extra

hormonal stimulation of the HC11-TRE-CTGF cells. There has not been previous evidence suggesting that CTGF/CCN2 may activate the transcription of β-casein in the absence of hormone; the considerable expression is most likely due to the effect of lactogens commonly present in the serum of the growth media. Subsequent experiments were therefore performed using charcoal-stripped serum in the growth media. However, the visible expression of β-casein mRNA in the absence of lactogenic hormones may indicate that the mechanism of CTGF/CCN2 is independent of dexamethasone or prolactin. The level of β-casein mRNA at 16 hours post stimulation was similar in both HC11-TRE and HC11-TRE-CTGF cells, suggesting that the effect of CTGF/CCN2 is seen rapidly after stimulation. This result indicates that enhanced expression of CTGF/CCN2 leads to earlier activation of the β-casein promoter than that which results from lactogenic hormone treatment alone.

Data is also presented in this thesis showing enhanced transcription of β-casein in primary mammary epithelial cells that had been infected with the CTGF/CCN2-encoded adenovirus (Figure 8). These results suggest that the effect of CTGF/CCN2 on lactogenic differentiation is not specific to the HC11 cell line. CTGF/CCN2 has been shown to contribute to the differentiation of various other cell types, such as periodontal ligament-derived cells (6), rhabdomyosarcoma cells (50), osteoblasts and osteoclasts (167, 237) and, most commonly, fibroblasts and myofibroblasts (94). Thus, it can be inferred from this data that an enhancement of CTGF/CCN2 in the mammary gland *in vivo* may contribute to the shift from a proliferative phenotype to the support of differentiation.

Elevated full length CTGF/CCN2 is required for the enhancement of β-casein transcription

As previously mentioned, CTGF/CCN2 is a secreted protein that can be cleaved by proteases, such as matrix metalloproteinases, resulting in the formation of biologically functional and diverse proteins (94). The differentiation effects of CTGF/CCN2 seen in various tissues have been characterized to specific domains. For example, the N-terminal region is said to be responsible for the differentiation of myofibroblasts (94), while the CT domain alone is said to be responsible for chondrocytic differentiation (111). I have provided evidence suggesting that it is the full length CTGF/CCN2 protein that is required for the level of β-casein enhancement that is seen in response to elevated expression of CTGF/CCN2 (Figure 5).

CTGF/CCN2 contributes to the activation of Stat5

As previously mentioned, Stat5 is the predominant transcription factor required for the transcription of β-casein (92). The data presented in this thesis shows that elevated expression of CTGF/CCN2 enhances and sustains both the phosphorylation and the nuclear translocation of Stat5 (Figures 6 and 7). Elevated activation of Stat5 is typically the direct result of enhanced activation of the prolactin receptor/Jak2 signaling pathway. Currently there is nothing in the literature suggesting that CTGF/CCN2 may directly activate the prolactin receptor or Jak2. Moreover, there is no data suggesting that CTGF/CCN2 can deactivate SHP1 or PTP1A/B, which, when activated, lead to the deactivation of the Jak2/Stat5 pathway. However, there have been studies depicting a role for the extracellular matrix in the prolactin-independent activation of Stat5. Since

CTGF/CCN2 is a known stromal mediator (81), and we have shown data that elevated CTGF/CCN2 expression results in the enhanced activation of Stat5, the possibility that CTGF/CCN2 plays a role in the activation of Stat5 through a matrix-associated event is likely.

In 1995, Streuli *et al*. determined that the Stat5 DNA binding activity was present only in extracts of mammary cells cultured on basement membrane, suggesting that the activation state of Stat5 is regulated, in part, by substratum-to-cell adhesion (247). For example, HC11 cells over-expressing the DDR1 collagen receptor display a higher level of Stat5 phosphorylation upon stimulation with collagen and prolactin compared to control cells (73). This elevation in Stat5 activation corresponded to an increase in β-casein transcription (73), similar to the results found by our study. In addition, mice expressing a mammary-specific knock down of β1 integrin also displayed a decreased activation of Stat5, which corresponded to a decrease in β-casein transcription compared to control mice (75, 188).

A current issue being investigated by those who study lactogenic differentiation is the link between extracellular matrix-induced signaling and the activation of the prolactin receptor signaling pathway, specifically the Jak2/Stat5 pathway (247). Since laminin is the predominant protein of the basement membrane, the signaling pathways downstream of its β1 integrin complex receptor must be considered.

Elevated expression of CTGF/CCN2 eliminates the requirement of matrix proteins for β-casein transcription

To address the question of whether CTGF/CCN2 has an effect on the cell:matrix interaction required for β-casein transcription, this thesis presents data that looks at the level of β-casein transcription in HC11 cells grown not only on tissue culture plastic, but also on fibronectin, collagen I, and Matrigel coated dishes. The results suggest that while elevated CTGF/CCN2 expression enhances the level of β-casein mRNA to a level significantly higher than in the control cells, the cells grown in the presence of matrix proteins do not display a significant enhancement in the transcription of β-casein compared to the cells grown only on plastic (Figure 9). This matrix-mediated increase is seen in the control cells, with the highest level of transcription present in the cells grown on Matrigel, of which laminin is the main component. These results imply that CTGF/CCN2 acts as a competitor for the laminin receptors on the epithelial cells. CTGF/CCN2 partially abrogates the requirement for these proteins and most likely contributes to the signaling pathways typically activated downstream of adhesion to the matrix. Overall, we can conclude from this data that CTGF/CCN2, in combination with lactogenic hormones, activates β-casein transcription to near the threshold level that can be achieved, regardless of added matrix proteins. This suggests that CTGF/CCN2 interacts with and activates β1 integrin to a greater extent than is achievable in the control cells.

Data is also presented in this research showing that CTGF/CCN2 enhances the expression of surface level α6 and β1 integrins (Figure 17), which, together as a complex, is a common receptor for laminin. These results confirm and extend our previous finding

of CTGF/CCN2-mediated enhancement of β1 integrin protein levels (271). This finding supports the idea that elevated CTGF/CCN2 expression can enhance the levels of α6 and β1 integrin activity and their subsequent influence on the activation of the β-casein promoter.

CTGF/CCN2 contributes to the binding of Stat5 to the β-casein promoter

Should the action of CTGF/CCN2 truly result in the phosphorylation of Stat5 and subsequent activation of the β-casein promoter, it would be expected that cells with elevated CTGF/CCN2 expression would display enhanced binding of Stat5 to the β-casein promoter compared to the binding in the control cells. Research presented in this thesis supports this idea. By chromatin immunoprecipitation, during which DNA is immunoprecpitated when bound to a DNA binding protein such as a transcription factor, it was found in this study that once treated with DIP, the HC11-TRE-CTGF cells display a higher level of Stat5 binding to the β-casein proximal promoter compared to the control cells (Figure 10).

Recently, Xu *et al*. found that human mammary epithelial cells, when stimulated with laminin-1 in the absence of prolactin, display a slightly enhanced binding of Stat5 to the β-casein proximal promoter, as well as to the γ-casein promoter (287). While Xu *et al*. showed that stimulation with both prolactin and laminin is necessary for the optimal binding of transcription factors and chromatin remodeling machinery to the casein promoters (287), results from our study demonstrated nearly optimal activation of β-casein transcription in the presence of CTGF/CCN2 without additional laminin protein. It may be possible that the increase in Stat5 binding with laminin-1 stimulation in the Xu

et al. study is amplified in the presence of elevated CTGF/CCN2 expression due to its interaction with the integrin signaling pathways required for activation of both Stat5 and the β-casein promoter.

The findings from this thesis so far suggest that CTGF/CCN2 expression contributes to the transcription of β-casein through the activation of β1 integrin-mediated signaling, leading to the activation of Stat5 and its subsequent activation of the β-casein promoter. The data presented in this study shows a CTGF/CCN2-mediated elevation of β-casein transcription in both HC11 and primary mouse mammary epithelial cells. This finding is supported by CTGF/CCN2-mediated enhancement of Stat5 activation, nuclear translocation, and DNA binding ability in combination with prolactin. CTGF/CCN2 is known to interact with various integrin complexes (34, 37, 85), and β1 integrin-mediated signaling is required for the activation of Stat5 (75, 248), and this study shows that CTGF/CCN2 enhances the surface level expression of β1 integrin and also competes for the matrix protein binding sites on the integrin during lactogenic differentiation. Together, these results support a contribution of CTGF/CCN2 to the activation of β1 integrin-mediated signaling leading to the activation of Stat5 and its subsequent activation of the β-casein promoter.

Recently, studies which investigate the mechanism of crosstalk between prolactin receptor signaling and integrin signaling leading to the activation of Stat5 have provided some new insights (2, 3, 128, 129). In 2006, a study demonstrated that Rac1, a Rho GTPase, is required downstream of integrins for activation of the prolactin receptor/Jak2/Stat5 signaling pathway (3), and this activation is required for β-casein synthesis in mammary epithelial cells. In the same year, another study performed in

mouse embryonic fibroblasts showed that Rac1 and a GTPase-activating protein, MgcRacGAP, bind directly to phosphorylated Stat5 and are required to promote its nuclear translocation (129). Most recently, it was found that the MgcRacGAP is a nuclear localization signal-containing nuclear chaperone of phosphorylated Stat5 (128), and also that in mammary epithelial cells, Rac1 is activated downstream of the focal adhesion complex, particularly downstream of ILK (integrin-linked kinase), possibly independently of FAK (2). Together, the data from these studies suggests that the formation of focal adhesion complexes downstream of β1 integrin signaling leads to the activation of Rac1, which is involved in the activation and nuclear translocation of Stat5 to the β-casein promoter. In our study, it was therefore important to determine the effect of CTGF/CCN2 on the signaling mechanisms downstream of β1 integrin in order to fully comprehend the mechanism of its effect on the transcription of β-casein.

The Contribution of CTGF/CCN2 to Cell Adhesion and Survival

The potential effect of CTGF/CCN2 on HC11 cell adhesion is important because integrin engagement and attachment to specific components of the ECM regulates prolactin-induced expression of milk proteins (155). Recently published data from our lab showed that the elevation of CTGF/CCN2 increased the protein expression of β1 integrin as well as the level of phosphorylated focal adhesion kinase (FAK) (271). FAK is a member of the focal adhesion complex that forms as a result of integrin clustering (36).

Upon the genetic deletion of β1 integrin, mammary epithelial cells undergo cell cycle arrest and the glands display defective development *in vivo*, suggesting that

mammary epithelial cell growth and survival requires integrin-mediated adhesion (186). A study by Faraldo *et al.* determined that the perturbation of β1 integrin function induced precocious dedifferentiation of the secretory epithelium in the mammary gland (75), suggesting that β1 integrin-mediated adhesion is particularly critical for the differentiation of mammary epithelial cells.

CTGF/CCN2 is known to be involved in matrix production and proliferation in concert with other growth factors in many cell types (80, 254), and directly involved in cell adhesion and differentiation in a variety of cell types (9, 193). The cell adhesion and differentiation effects mediated by CTGF/CCN2 are commonly found to be the result of the interaction between CTGF/CCN2 and integrin complexes (7, 34, 35, 85, 87). The data presented in this thesis supports these findings and our hypothesis that CTGF/CCN2 acts as a regulator of cell-matrix adhesion and survival through the stabilization of β1 integrin-mediated signaling.

CTGF/CCN2 sustains the proliferation and survival of HC11 cells

In order to truly understand the effect of CTGF/CCN2 on β1 integrin-mediated events such as cell adhesion and survival, assays were performed on cells grown in the absence of serum. Typically in the absence of serum, which contains many growth factors necessary for the cells to adhere and survive such as serum albumin and platelet-derived growth factor (PDGF), epithelial cells will lose their integrin-mediated attachment and undergo an apoptotic process called anoikis. Performing survival assays in the absence of serum thereby allows for the analysis of adhesion-specific mechanisms.

Data presented in this thesis demonstrated CTGF/CCN2-mediated sustainment of HC11 cell survival through 96 hours in the absence of serum by MTT assay (Figure 11). The MTT assay specifically detects cells that are metabolically viable. The ability of the cells to convert the MTT dye into a colorimetric water-soluble formazan salt is quantified by reading the absorbance. Both HC11-TRE-CTGF cells grown in growth media, and HC11-TRE control cells grown in CTGF/CCN2-containing media, showed sustained and even slightly enhanced numbers of viable cells after 96 hours of incubation in serum-free conditions. Thus, this data shows that enhanced expression of CTGF/CCN2 maintains the integrin-mediated adhesions required for the sustained viability of HC11 cells. Similar survival effects of CTGF/CCN2 have been shown in endothelial cells and rhabdomyosarcoma cells also deprived of growth factors (7, 50).

The question may then be raised as to whether the effect seen in the MTT assays is truly the result of an effect of CTGF/CCN2 on integrin-mediated adhesion. CTGF/CCN2 has been shown to interact directly with integrins and enhance adhesion in breast cancer cells (37), as well as endothelial cells (7), skin and gingival fibroblasts (34, 102), hepatic stellate cells (84, 85), chondrocytes (192), and oval cells (206). Many matricellular adhesion proteins contain the tripeptide arginine-glycine-aspartic acid (RGD) as their cell recognition site (222). The RGD sequences of these proteins, such as laminin or collagen, are recognized by the protein receptors, specifically integrins (222). The addition of an RGD-containing peptide resulted in a diminished response to CTGF/CCN2, suggesting the RGD peptide is competing for the site on the surface level integrins that recognizes CTGF/CCN2 (Figure 18). This data implies that the survival effect arbitrated by CTGF/CCN2 is the result of an interaction with integrins.

CTGF/CCN2 allows HC11 cells to move through the cell cycle

As previously mentioned, the disruption of cell-matrix attachment results in a loss of pro-survival signals, and this loss ultimately results in the process of anoikis. Mechanisms that regulate the cell cycle and apoptosis are closely linked (44). Data is presented in this thesis supporting this notion. Under serum-free conditions, elevated expression of CTGF/CCN2 not only maintains the integrin-mediated survival of mammary epithelial cells, but also allows the cells to continue to move through the cell cycle, whereas a higher population of control cells appear in sub-G_0, indicative of possible apoptosis.

In flow cytometry-based cell cycle analysis, the cells are sorted based on their DNA content, such as normal diploid (G_0/G_1) or aneuploid which could be either cells undergoing DNA replication (S) or mitosis (G_2/M). Typically, normally growing cells will show the greatest population of cells in the G_1 phase, less in the G_2/M phase, and few in the S phase. Under serum-free conditions, this remained true for both the HC11-TRE and HC11-TRE-CTGF cells, yet the HC11-TRE-CTGF cells showed a greater percentage of cells in the S phase compared to the HC11-TRE control cells. This data supports the finding from the MTT assay and suggests that the mechanism of CTGF/CCN2 is allowing the cells to continue to grow and synthesize DNA even in the absence of serum. CTGF/CCN2 has been shown to control cell cycle progression through late G1 and S-phase entry in NRK fibroblasts suspension cultures (142). In the fibroblast system, TGFβ-regulated CTGF/CCN2 induces S-phase entry through the downregulation of the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$, ultimately enhancing cyclin A levels (142).

In this thesis, I have presented data showing a CTGF/CCN2-mediated enhancement of cyclin D1 in HC11 cells. Cyclin D1 is important during the early to mid-G_1 phase of the cell cycle. It associates with Cdks 4 and 6, which are partially responsible for the inactivation of the retinoblastoma protein (pRB), which when active binds to and inhibits the E2F family of transcription factors that drive the cells into S-phase (249). A similar effect of enhanced expression of CTGF/CCN2 has also been shown in esophageal squamous cell carcinoma cells (59). In this study by Deng *et al*., a high level of CTGF/CCN2 expression was associated with enhanced growth of the tumor cells, primarily due to the ability of CTGF/CCN2 to increase the expression of cyclin D1 and c-myc downstream of mitogenic T-cell factor/lymphoid enhancer factor (Tcf/Lef) signaling (59). Also, CTGF/CCN2 was shown to enhance the level of cyclin D1 in human lung fibroblasts via the downregulation of $p27^{Kip1}$(281), resulting in enhanced cell proliferation and progression through the cell cycle. Together with the data presented in this research, these findings support a role for CTGF/CCN2 as a regulator of cell cycle progression in multiple tissue types.

CTGF/CCN2 protects mammary epithelial cells against anoikis

As previously mentioned, cells found in the sub-G_0/G_1 phase of the cell cycle are thought to be undergoing apoptosis, revealed by the decreased amount of intact DNA present in apoptotic cells. It is known that upon the deletion of β1 integrin, mammary epithelial cells tend to undergo cell cycle arrest and the mammary epithelial cells proliferation and survival requires integrin-mediated ECM adhesion (156). That these

cells were maintained in the absence of serum warranted the investigation into the level of apoptosis seen in the HC11-TRE cells.

Data is presented in Figures 14 and 15 showing the tendency of HC11-TRE control cells to undergo apoptosis both by TUNEL assay and by looking at the percentage of PARP cleavage in both cell lines. The data shows that HC11-TRE-CTGF cells do not succumb to the same fate, suggesting that CTGF/CCN2 is able to protect the epithelial cells from undergoing anoikis.

The absence of survival signaling leads to an imbalance in the ratio of anti-apoptotic factors to pro-apoptotic factors (210). Pro-apoptotic factors such as Bax or Bid form homodimers in the mitochondrial membrane leading to the release of cytochrome c and subsequent activation of the caspase cascade (91). Detachment of mammary epithelial cells from ECM proteins has been shown to trigger the translocation of cytoplasmic Bax to the mitochondrial membrane (91). The effect of CTGF/CCN2 on apoptosis is clearly seen by the TUNEL assay, which labels fragmented DNA and confirms that cells are in a state of late apoptosis. The data presented shows far more HC11-TRE control cells staining positive for TUNEL compared to the HC11-TRE-CTGF cells, suggesting that in the absence of serum the cells with enhanced expression of CTGF/CCN2 continue to thrive.

PARP, or poly(ADPribose) polymerase, is cleaved by caspases during the apoptotic process. Cleaved PARP then attempts to repair any DNA damaged in the process and depletes the cell of ATP, which ultimately causes the cells to lyse and die. An enhancement of cleaved PARP, as is seen in the HC11-TRE cells after being maintained for 4 days in the absence of serum, confirms and extends the finding that the

cells without elevated CTGF/CCN2 expression undergo apoptosis in the absence of serum and integrin-mediated attachment.

CTGF/CCN2 has been shown to protect against apoptosis in human rhabdomyosarcoma cells (50). In this study, the loss of cell growth that occurred as a result of anti-sense CTGF/CCN2 was characterized by a significant increase in apoptosis (50). In human mesangial cells, CTGF/CCN2 was found to ultimately stabilize the levels of the anti-apoptotic protein Bcl-2 through the activation of MAP kinase phosphatase-1 (Mkp1) (264). Mkp1 is responsible for the inactivation of p38 MAP kinase and c-jun kinase (JNK), which phosphorylate and deactivate Bcl-2 under stress-induced apoptotic conditions.

In human renal epithelial cells (164), rat aortic smooth muscle cells (72), normal rat kidney fibroblasts (142), and human mesangial cells(105), CTGF/CCN2 was shown to dose-dependently enhance cell viability by the activation of growth and survival factors Erk1/2 and Akt, as well as the suppression of apoptotic factors such as caspase 3 (72). In contrast, CTGF/CCN2 may induce apoptosis in renal pericytes (160) and MCF7 human breast cancer cells (107). While it is difficult to determine whether apoptosis is a physiologically relevant function of the protein due to the fact that these cells are typically treated with very high concentrations, CTGF/CCN2 is known to accumulate in the local extracellular environment, thus the possibility cannot be excluded (93). In support of the current body of evidence suggesting that CTGF/CCN2 supports survival, the data presented in this thesis favors the activity of CTGF/CCN2 as being anti-apoptotic in HC11 cells under serum-free conditions.

Elevated CTGF/CCN2 enhances β1 integrin – FAK-mediated adhesion signaling

Recently published data from our lab showed that the elevated expression of CTGF/CCN2 in HC11 cells enhances not only the level of β1 integrin protein expression, but also the level of phosphorylated FAK (271), whose activation is indicative of adhesion-mediated survival signaling (36). In human intestinal epithelial cells, it was found that the inhibition of FAK activation resulted in detachment of the cells and subsequent anoikis (19). In the mammary gland, conditional knockout of FAK resulted in decreased proliferative capacity of the epithelial cells mediated by downregulation of MAP kinase activity and cyclin D1 (186).

Data is presented in this thesis that confirms and extends our previous findings of CTGF/CCN2-mediated enhancement of β1 integrin and phosphorylated FAK, to now an increase in the levels of cyclin D1 and phosphorylated Akt. CTGF/CCN2 $^{-/-}$ cells have been shown to display a decrease in phosphorylated FAK and Akt (192, 235). Elevated CTGF/CCN2 has conversely been shown to lead to the phosphorylation of Akt (49, 100). The treatment of cardiac myocytes with both full length and C-terminal region peptides of CTGF/CCN2 and subsequent elevation of activated Akt resulted in cardiac hypertrophy (100). In retinal neovascular disease-associated fibrosis, the elevation of CTGF/CCN2 in bovine retinal capillary cells also resulted in enhanced activation of the PI3K-Akt pathway (250), suggesting that this growth and survival effect mediated by CTGF/CCN2 has implications in multiple diseased states.

CTGF/CCN2 contributes to the formation of focal adhesions

A critical component of epithelial cell survival is the ability of the cells to adhere to their surrounding matrix. This adhesion is commonly mediated by the association of cell-surface αβ integrin complex clusters with matrix proteins such as laminin, fibronectin, or collagen. The cell:matrix interaction stimulates the transmission of survival signaling through the activation of proteins associated with the focal adhesion complex, including a variety of structural (i.e., vinculin), adaptor (i.e., paxillin, p130cas, parvin), and enzymatic proteins [i.e., focal adhesion kinase (FAK), integrin-linked kinase (ILK), Src] (28).

The principle matrix protein in the basement membrane upon which mammary epithelial cells reside and with which it interacts is laminin (155, 248). The α6β1 integrin complex is a known laminin receptor on mammary epithelial cells (62). This complex is also known to be a receptor for CTGF/CCN2 in human fibroblasts (102, 116, 154). As mentioned previously, Figure 17 of this thesis shows a CTGF/CCN2-mediated enhancement of α6 and β1 integrins on the surface of HC11 mouse mammary epithelial cells by flow cytometry. This suggests that the survival-related effects elicited by CTGF/CCN2 are mediated through α6β1 integrin-containing adhesion complexes. While other integrins are expressed on the surface of HC11 cells (62) and CTGF/CCN2 has been shown to interact with other integrin complexes (84, 85, 192), laminin is the primary matrix component for the cell type being studied, thus making its receptor of great importance. Based on the data suggesting that CTGF/CCN2 partially abrogates the need for laminin for lactogenic differentiation, the α6β1 laminin receptor complex was investigated.

The formation of integrin clusters leads to the development of focal adhesion complexes as described previously, and data is presented here showing a CTGF/CCN2-mediated enhancement of multiple focal adhesion complex-associated proteins. Data also shows greater development of focal adhesions in HC11-TRE-CTGF cells by the immunofluorescent staining of vinculin, compared to the HC11-TRE control cells.

A study by Chen *et al*. provided evidence that, in fibroblasts, CTGF/CCN2 induces extensive and prolonged formation of filopodia and lamellipodia, and this corresponds to the formation of α6β1 integrin-containing focal adhesion complexes localized at the leading edges of pseudopods (34). The data from this thesis (Figures 19 and 20) supports these findings in mammary epithelial cells, suggesting that this effect of CTGF/CCN2 is not cell type specific. Also, in fibroblasts, CTGF/CCN2 was shown to induce the activation of intracellular signaling molecules FAK and paxillin (34), similar to our findings in the mammary epithelial cells.

It has been shown that CTGF/CCN2 contributes to the recruitment of Src to the focal adhesion complex through the enhanced activation of FAK (48), and this association ultimately results in the activation of Akt downstream of the focal adhesion complex. In mice with a Src deletion, the mammary glands display defective ductal development (134), suggesting that the activation of the FAK/Src-Akt signaling pathway is a critical pathway of epithelial cell survival.

HC11 cells adhere to CTGF/CCN2 via the α6β1 integrin complex

Thus far the data presented has shown that CTGF/CCN2 can contribute to cell survival through the formation of focal adhesion complexes. In order to determine

whether the formation of these complexes actually resulted in enhanced cell adhesion, assays were performed by coating tissue culture plates with CTGF/CCN2 and allowing control HC11-TRE cells to adhere. HC11-TRE cells displayed enhanced adhesion to the CTGF/CCN2-coated wells compared to the uncoated wells. To further confirm the mechanism of CTGF/CCN2 enhancing adhesion through the α6β1 integrin complex, the same adhesion assays were performed in the presence of function blocking antibodies to both integrins. CTGF/CCN2-mediated enhancement of adhesion was decreased to the level of adhesion to control wells in the presence of the function blocking antibodies, confirming that CTGF/CCN2 does exert its effects through the α6β1 integrin complex.

In a recent study by Leu *et al*., a similar technique was employed to determine the interaction of both CYR61/CCN1 and CTGF/CCN2 with the α6β1 complex in primary human skin fibroblasts (154). The results from that study support the finding from our study suggesting an interaction between CTGF/CCN2 and the α6β1 complex resulting in enhanced cell adhesion. The study by Leu *et al*. also determined a binding site for α6β1 in the TSP-1 domain of both CYR61/CCN1 and CTGF/CCN2, as well as in NOV3/CCN3 (154), confirming a possible interaction between the CCN proteins and the common laminin receptor.

The findings from this thesis advocates CTGF/CCN2 as a regulator of the survival of mammary epithelial cells by contributing to the stabilization of surface level integrin clusters and subsequent development of focal adhesions and resulting adhesion-mediated survival signals. This overall effect of CTGF/CCN2 has also been demonstrated in various cell types (192, 231). It was found in these studies that a reduction in the expression of CTGF/CCN2 corresponded to decreased levels of surface level integrin

expression, activated FAK and activated Erk1/2 (192, 231). In human skin fibroblasts, CTGF/CCN2 induces extensive formation of α6β1 integrin complex-containing focal adhesions and enhancement of subsequent adhesion signaling through FAK, paxillin, and Rac1 (34). These findings suggest that CTGF/CCN2 exerts its effects on the development of various cell types by engaging integrins and activating integrin-dependent signaling pathways (192), similar to the findings suggested by the data presented here in the HC11 mammary epithelial cells. Since CTGF/CCN2 is a known integrin-binding protein (7, 21, 37), the results presented on the role of CTGF/CCN2 in mammary epithelial cells concur with the data in the current literature suggesting that CTGF/CCN2 acts as a regulator of cell adhesion and survival in multiple cell types.

CTGF/CCN2 Contributes to the Transcriptional Regulation of Genes Relevant to its Function as a Regulator of Cell Adhesion and Survival During Lactogenesis

Genes regulated by CTGF/CCN2 are similar in fibroblasts and HC11 cells

Results from a genome-wide mRNA expression analysis on the HC11-TRE and HC11-TRE-CTGF cells support the role for CTGF/CCN2 as a regulator of adhesion and survival (**Tables 1 and 2**). The results are supported by the work of Kennedy *et al.*, who determined that *ctgf*/*ccn2*-/- mice displayed significantly reduced expression of pro-adhesive, pro-inflammatory and pro-angiogenic genes (132). Our microarray analysis determined that HC11-TRE-CTGF cells express significantly elevated levels of pro-adhesive and pro-proliferative genes. Pro-adhesion genes that were reduced in the mouse embryonic fibroblasts and elevated in HC11-TRE-CTGF cells included T-cadherin, R-

cadherin, fibronectin 1, laminin α5 and β2, thrombospondin 1 and syndecan 4. While fibronectin 1 and the laminin subunits are known matrix proteins found in the basement membrane surrounding mammary epithelial cells, and cadherins are known membrane-associated adhesion molecules, the regulation of both thrombospondin 1 and syndecan 4 by CTGF/CCN2 is also significant. Syndecan 4 is a heparin sulfate chain-associated molecule used by CTGF/CCN2 to enhance the adhesion of both mammary epithelial cells and fibroblasts to fibronectin (38, 57), as well as to facilitate the binding of growth factors to their cell surface receptors. Thrombospondin 1 is a glycoprotein that is incorporated into the ECM of both fibroblasts and mammary epithelial cells and is known to facilitate adhesion (174). CTGF/CCN2 is also a stromal mediator protein and contains a thrombospondin-1 sequence homology domain in its C-terminal region, thus the two proteins are commonly investigated simultaneously, though their functions are not always the same (265). Furthermore, the functions attributed to the TSP-1 domain of CTGF/CCN2 are not always consistent with those of thrombospondin-1 (255), yet the expression of both proteins is under regulation of TGFβ in various conditions (214).

CTGF/CCN2 transcriptionally regulates adhesion-related genes

The mRNA expression analysis also revealed the significant upregulation of various other adhesion-related genes in HC11-TRE-CTGF cells. Of particular interest are two members of the Rho GTPase family, RhoD and RhoU. RhoD is known to play a role in the activation of Src-mediated invasion signals in both kidney and colonic cancers (191). A homolog of the focal adhesion-activated protein cdc42, RhoU, which is an atypical Rho GTPase in that it is constitutively bound to GTP, has been shown to localize

to focal adhesions and to contribute to Src-mediated podosome formation in mammary epithelial cells (225). It is also known to stimulate quiescent mammary epithelial cells to re-enter the cell cycle (225). Both the formation of focal adhesions and cell cycle progression were also properties attributed to elevated expression of CTGF/CCN2 in HC11 cells in the results shown in this thesis.

Also of interest are the protein tyrosine phosphatase receptor type κ (Ptprκ) and the thyroid hormone receptor interactor 6 (Trip6). Ptprκ is a mediator of TGFβ in mammary epithelial cells and is known to contribute to the sustained activation of both FAK and Src and the resulting formation of focal adhesions (269). Also a focal adhesion-associated protein, Trip6 is a LIM domain protein that is essential for FAK phosphorylation and subsequent mature focal adhesion formation in HeLa cells (8, 63). The contribution of CTGF/CCN2 to the transcriptional regulation of the genes encoding these proteins strongly supports CTGF/CCN2 as a mediator of focal adhesion formation and resultant cell adhesion.

CTGF/CCN2 transcriptionally regulates mitogenic and survival genes

In addition to extracellular matrix signaling-associated genes, the microarray analysis showed that CTGF/CCN2 regulates the transcription of many genes involved in mitogenic cellular functions, including four genes of the dual specificity protein phosphatase (Dusp) family. Of importance, Dusp1 and Dusp16 are common members of the MAP kinase phosphatase family (MKP) of proteins, Mkp1 and Mkp7, respectively. These proteins act to specifically dephosphorylate and deactivate p38 MAP kinase and JNK (133), and thus are known to be pro-survival factors. It has recently been shown

that CTGF/CCN2 mediates the survival of human mesangial cells via the upregulation of Mkp1 and subsequent inactivation of stress protein p38 MAP kinase (264). Mkp7 is mitogenic and is stabilized by Erk activity, resulting in the inhibition of JNK. The Dusp3 gene, which is also regulated by CTGF/CCN2, is critical to cell cycle progression in cervical epithelial cells (103). CTGF/CCN2-mediated regulation of the Dusp genes may be another mechanism by which it sustains progression through the cell cycle under stress conditions, such as the absence of serum.

Microarray analysis also shows CTGF/CCN2-mediated regulation of a series of genes of the EGF-family, including amphiregulin, epigen, and epiregulin. Amphiregulin is a heparin-binding peptide that has been shown to contribute to mammary epithelial cell growth and proliferation (238). In order to become activated, it must be cleaved from its original transmembrane form by ADAMTS proteins (242), which were also shown to be regulated by CTGF/CCN2 by the microarray analysis. It has also been shown to be upregulated simultaneously with CTGF/CCN2 in response to the interaction between Factor VIIa and tissue factor in keratinocytes, implicating both proteins in wound repair (29). Epigen is known to stimulate the growth of epithelial cells through Erk activation, though its binding affinity for the EGF receptor is 100-fold less than EGF (138, 244). Finally, epiregulin, a common ligand for both the EGF receptor and ErbB4, is commonly expressed in most breast cancers as a result of deregulated proliferative phenotype (124, 213). It has been suggested that there may be cross-talk between integrin signaling and EGF receptor-mediated signaling, and the transcriptional upregulation of these EGF family members by CTGF/CCN2, an integrin-binding protein, may contribute to that integration.

Other factors transcriptionally regulated by CTGF/CCN2 in HC11 cells that are responsive to EGF-induced signaling include the small GTPase RhoB, as well as a member of the AP-1 family of transcription factors, JunD. RhoB is highly expressed in MCF7, T47D, and immortalized mammary epithelial cells, such as HC11 cells (55). It has been suggested that RhoB plays an important role in the EGF-stimulated growth of mammary epithelial cells, both normal and cancer cells (55). JunD, as a member of the AP-1 transcription factor complex, is also known to contribute to the enhancement of mammary epithelial cell proliferation as a result of treatment with EGF (175). Results from this thesis determined that elevated expression of CTGF/CCN2 enhances cell proliferation in the presence of EGF, thus the results from the microarray analysis confirm the mitogenic role of CTGF/CCN2 in HC11 cells, as well as suggests new avenues down which CTGF/CCN2 may be exerting its effects, such as the integration of integrin- and EGF-mediated signaling pathways.

While we have successfully shown CTGF/CCN2-mediated enhancement of phosphorylated Akt, a survival mediator protein, microarray analysis has also shown CTGF/CCN2-mediated transcriptional regulation of genes associated with the transcription factor NF-κB. Signaling through the NF-κB pathway is also another mechanism by which integrin-mediated signaling results in cell survival (46). Two proteins important for this process, RelB and Traf1, were shown to be transcriptionally regulated by CTGF/CCN2. TNF-associated factor 1, Traf1, is an anti-apoptotic protein regulated by NF-κB particularly in breast cancer cells (201). RelB is a protein that complexes with the p52 subunit of Nf-κB, and the complex is induced by tumor necrosis factor (TNF), resulting in the binding of the complex to the cyclin D1 promoter in

mammary epithelial cells (295). RelB-containing complexes are commonly observed in mouse mammary tumors induced by carcinogen exposure, and RelB has recently been found to be synthesized *de novo* in invasive estrogen receptor-negative (ER-) breast cancer cell lines, resulting in sustained stabilization of anti-apoptotic Bcl-2 (272). NF-κB commonly regulates the expression of genes involved in cancer cell invasion, metastasis, angiogenesis, and resistance to chemotherapy downstream of integrin complex activation (190), thus the regulation of NF-κB-associated factors may be a mechanism by which CTGF/CCN2 may be involved in cancer progression.

CTGF/CCN2 transcriptionally regulates genes involved in mammary gland development and cancer

As previously mentioned, evidence has been provided showing that CTGF/CCN2 is elevated in a number of cancers, including pancreatic (275), breast (285), glioblastoma (198), esophageal (139), melanoma (144), acute lymphoblastic leukemia (263), and hepatocellular carcinoma (293). These studies implicate CTGF/CCN2 in both the development and progression of the disease. Of particular interest to breast cancer, the microarray analysis showed CTGF/CCN2-mediated regulation of many genes also known to be involved in the progression of the disease. Some of these genes include the urokinase receptor, fibroblast growth factor 13 (Fgf13), epiregulin, artemin, RelB, transcription factor E2F2, and polo-like kinase 3 (Plk3). These are known to be oncogenic factors in breast cancer cells, particularly in regards to uncontrolled proliferation (166, 213, 234, 272, 284), and enhanced metastatic abilities (126, 157, 172), supporting a role for CTGF/CCN2 in the development and progression of breast cancer.

Genes encoding proteins known to be specifically involved in mammary gland development were also shown to be regulated by CTGF/CCN2 by the global mRNA expression analysis. These proteins included the tissue inhibitor of metalloproteinase 3 (TIMP3), inhibin βA, fibroblast growth factor 13 (Fgf13), amphiregulin, Wnt4, RelB, and tumor necrosis factor receptor superfamily 12a (Tnfrsf12a). Fgf signaling is required for the earliest stage of mammary gland development, the formation of a mammary placode (166), though Fgf13, specifically, has not yet been classified as required for mammary gland development. The Fgf receptors are highly expressed in proliferating and invading terminal end buds (TEBs) in the mouse mammary gland (166), suggesting a role for Fgf signaling during early gland development. Amphiregulin, as a regulator of growth via its interaction with the EGF receptor, is required for the epithelial-mesenchymal cross-talk necessary for postnatal mammary gland development (242). Wnt4 and Tnfrsf12a are both known to stimulate ductal branching in virgin and pregnant mice, Wnt4 as a mediator of progesterone signaling (23), and Tnfrsf12a as a target of the transcription factor Runx2 (173, 208), which is also a known regulator of β-casein transcription (117). During pregnancy, RelB, in complex with the p52 subunit of NF-κB, contributes to ductal branching morphogenesis by facilitating the induction of cyclin D1 activity (58). Finally, TIMP3 and inhibin βA are required for successful lactation (77, 217). In the absence of TIMP3 in the mouse mammary gland, there is evidence of lumen collapse, epithelial apoptosis, and a loss of epithelial-alveoloar architecture (77), all of which are requirements for lactogenesis. Loss of inhibin βA in mice results in lactation failure due to the retardation of ductal elongation and alveolar morphogenesis (217). The regulation of these genes required for various stages mammary gland development by CTGF/CCN2

supports the data from this thesis suggesting that CTGF/CCN2 is present at all of the developmental stages, with the majority of function being in the lactogenic process that occurs during late pregnancy.

Conclusion

The first part of this study confirmed that CTGF/CCN2 expression contributes to the transcription of β-casein through the activation of β1 integrin-mediated signaling leading to the activation of Stat5 and its subsequent activation of the β-casein promoter. The second component established that the mechanism of CTGF/CCN2 in mammary epithelial cells is to regulate survival by contributing to the stabilization of surface level integrin clusters and subsequent development of focal adhesions and resulting adhesion-mediated survival signals. Global expression profiling of HC11-TRE-CTGF cells provided additional insight into these processes. Together, the research presented in this thesis affirms my hypothesis that CTGF/CCN2-mediated stabilization of the adhesive properties of the mammary epithelial cells leads to the activation of the β-casein promoter and subsequent transcription of the milk protein when the cells are stimulated to undergo lactogenic differentiation.

One question that arises is how exactly does the CTGF/CCN2-mediated enhancement of integrin-mediated adhesion signaling contribute to the lactogenic differentiation process that includes the transcription of β-casein. Early in this study, we found that CTGF/CCN2 is most highly expressed in the mammary gland late in pregnancy, and sharply decreases after parturition and the initiation of lactation. The pregnant mammary gland is characterized by proliferating epithelial cells that form the

many alveolar structures that fill the mammary gland, and the subsequent survival of those cells waiting to undergo differentiation in response to lactogenic hormones is required for successful lactation. In cell culture, the process of cell proliferation required for response to hormones is referred to as competence (31). Data presented here shows that CTGF/CCN2 has the ability to enhance the proliferation and survival of mammary epithelial cells. While the mammary gland and its alveolar structures are rapidly expanding, some epithelial cells may lose full attachment to the basement membrane. In theory, this should result in cell death, but the data presented in this thesis showed that CTGF/CCN2 is effective under conditions where epithelial cells lose their attachment to matrix proteins. Thus *in vivo*, epithelial cells expressing CTGF/CCN2 may have the ability to stabilize the integrin complexes upon detachment and thus survive until they can reattach to the basement membrane.

In relation to the activation of β-casein transcription, there has been debate about whether the adhesion of the cells to the matrix or to one another is the most critical for activation of the promoter. In 1991, Streuli *et al*. placed mammary epithelial cells into a suspension culture assay where the cells would not interact with each other and stimulated them with lactogenic hormones (246). This study showed that the cells grown in the laminin-based media were able to produce β-casein and this interaction with laminin was mediated by integrins, yet cells grown in collagen-based media required the interaction with other cells in order to produce β-casein (246). Subsequently, it was found that signals required for the transcription of β-casein were inhibited by the addition of blocking antibodies to both α6 and β1 integrins, as well as the E3 laminin receptor (183). Together with the data presented in this thesis, these findings support the idea that

CTGF/CCN2 acts on epithelial cells during pregnancy to maintain their integrin-mediated signaling pathways that not only lead to survival, but also the activation of β-casein.

Recently, mammary gland knockout studies have ascertained the effects of β1 integrin as well as FAK and Src on the development of the mammary gland, including lactogenesis (74, 186, 273). Mammary specific inhibition of β1 integrin resulted in reduced activation of MAPK and Akt, and ultimately in growth defects (74). Similarly, a mammary specific knockout of FAK resulted in growth defects as well as a reduction in milk protein production, due to decreased proliferative capacity of the secretory epithelium and lack of Stat5 activation (186). A mammary-specific knockout of Src resulted in a lack of lactational ability, but retained normal growth of the mammary gland (273). In this study by Watkin *et al.*, a reduction in expression of the prolactin receptor on the surface of mammary epithelial cells was implicated in the inability of the mammary gland to undergo lactogenesis. Thus, based on the results obtained from our lab and others, the effects of a mammary specific knock down of CTGF/CCN2 may prove useful in understanding the exact mechanism of lactogenic differentiation *in vivo*.

Significance

Full comprehension of the basic science behind the normal development of a tissue is critical for understanding the mechanisms involved in the diseased state of the same tissue and perhaps of other related tissues. In the mammary gland, development includes multiple phases of proliferation and death, not only of the epithelial cells, but also of the cells of the surrounding microenvironment such a fibroblasts, macrophages, and adipocytes (148, 241). Alterations in the mechanisms involved in these processes

may adversely affect the breast. Thus, investigation into the functions of the molecules involved in each phase of development is crucial in understanding the molecular basis for diseases of the breast, including but not limited to breast cancer.

Data presented in this thesis delineates a role for CTGF/CCN2 as a critical factor in lactogenic differentiation through its function as a regulator of integrin-mediated epithelial cell proliferation and survival during late pregnancy. While lactation deficiencies are rare and may be minor concerns in areas with adequate economic resources due to the availability of formula-based nourishment, it poses a grave concern for areas where the only source of nourishment and immune protection for an infant is the mother's breast milk (260). The research reported here has highlighted multiple factors required for proper mammary gland development during pregnancy and lactation. While it has been previously presented that β1 integrin and downstream FAK are required for functional differentiation of the epithelial cells (74, 186), this study now shows that CTGF/CCN2 is required upstream of these factors in order for the expression of the predominant milk protein, β-casein. These findings may provide evidence critical to solving the problem of lactation deficiencies and ensuing newborn fatalities.

The role of the tumor microenvironment has become a primary focus of breast cancer research (78). While the current study has focused on the role of CTGF/CCN2 being produced by epithelial cells and acting on epithelial cells, it is known to be secreted by fibroblasts as well *in vivo* (7). In the mammary gland, the epithelial cells take their prompts from the stromal microenvironment to orient themselves in regular structures surrounded by a basement membrane (148). In the normal gland, there is relatively little connective tissue surrounding the structures, while the tumor stroma contains copious

connective tissue as a result of the enhanced release of growth factors by the fibroblasts. Data has been presented here suggesting that secreted CTGF/CCN2 enhances the proliferation and survival of the mammary epithelial cells, and this may be one reason why CTGF/CCN2 is found at elevated expression levels in mammary tumors (285) .

As mammary tumors grow due to the deregulation of proliferation and angiogenesis, the alveolar structures expand similarly to the expansion seen in the pregnant gland (24). If the proliferation rate of the epithelial cells exceeds that of the formation of a basement membrane, the cells may lose their integrin-mediated attachment to matrix proteins that is required for the cells to survive. Similar to its function in the pregnant gland, CTGF/CCN2 expression may partially abrogate the need for matrix proteins by stabilizing the integrin complexes and the resulting downstream survival signals. In fact, Chen *et al*. (37) found that the elevated expression of CTGF/CCN2 in MCF7 breast cancer cells resulted in focal adhesion complex aggregation, similar to that seen in the HC11 cells.

The formation of focal adhesions correlates with the ability of cells to spread and migrate (51) due to the ability of the complex-associated proteins to interact with and alter the organization of the actin cytoskeleton. CTGF/CCN2 has been shown to be elevated in advanced stages of breast cancer in which the cells are highly migratory and metastatic (37, 127). Based on the results from this thesis, it is likely that the contribution of CTGF/CCN2 to the formation of focal adhesions under starvation conditions is a mechanism by which it may enhance the metastatic ability of breast cancer cells.

In summary, there are many factors that are critical to normal non-tumorigenic cellular functions that also play roles in advanced stages of cancer. Thus, it is imperative that we understand the molecular mechanisms in which those factors are involved, in order to fully understand and abrogate the tumorigenic process. This study provides compelling evidence that CTGF/CCN2 contributes to the integrin adhesion-mediated survival of mammary epithelial cells. It is, therefore, important to comprehend and appreciate the significance CTGF/CCN2, a molecule recently shown to be involved in both the normal process of lactogenic differentiation (271) as well as advanced metastatic breast cancer (37). In the future, it will be imperative to confirm our findings both in 3-D models of mammary acini, as well as in conditional mammary-specific knockouts of CTGF/CCN2. Understanding the mechanisms of CTGF/CCN2 in mammary epithelial cells will ultimately lead to the unambiguous comprehension of diseases of the breast.

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